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Successes and challenges in the developmemt of baculovirus-based vaccines for swine influenza and porcine circovirus type 2b

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Successes and challenges in the development of baculovirus-based vaccines for swine influenza and porcine circovirus type 2b

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CHAPTER 1: GENERAL INTRODUCTION

Introduction

Respiratory diseases present a major problem for the swine production industry in the United States (US) and globally. A report from the USDA in 2006 determined that nearly half of the reported pig deaths in the nursery phase and more than half of the deaths in the grower/finisher phase in 2006 were the result of respiratory disease mediated by viral and bacterial pathogens [1]. These results demonstrated a 10-20% increase in deaths associated with respiratory disease since 1990. The losses of animal inventory coupled with the expenses for pharmaceuticals and vaccines to combat the responsible pathogens results in a substantial economic impact on the swine industry as demonstrated by the nearly 500 million dollars in losses reported for a single respiratory pathogen, porcine reproductive and respiratory syndrome virus (PRRSV), in 2005 [2].

As the trend toward consolidated swine operations continues with over 50% of the US pig inventory in operations with 5000 head or more as of 2006, respiratory disease manifestations have become increasingly multifactorial in nature, potentially due to the increase in variability in immune status of pigs on large consolidated sites [1;3]. Labeled the porcine respiratory disease complex (PRDC), multifactorial respiratory disease in pigs has been associated with a variety of viral and bacterial pathogens [4;5]. Affecting mostly grower/finisher pigs, this disease syndrome is characterized by severe symptoms of respiratory distress including coughing, labored breathing, and lethargy in addition to fever, and inappetence leading to decreased weight gain [6]. Outbreaks typically occur in 15 to 22 week old pigs but can occur in younger pigs presumably due to the environmental stress of shipment and mixing with new cohorts after movement from nursery operations [7;8]. Morbidity and mortality associated with PRDC are highly variable and

dependent on the etiological agents responsible for the outbreak as well as the age and immune status of the affected animals [3]. Due to the complexity of the infections, the disease symptoms can be quite prolonged, leading to increased production costs associated with extended finishing timelines and the need for medications.

Swine influenza virus (SIV) and porcine circovirus type 2 (PCV2) are two of the disease agents commonly associated with PRDC [4;9]. Although SIV is considered a secondary infection in the etiology of PRDC, recent reports of persistence in swine herds suggest an increased prevalence of this virus setting the stage for more frequent co-infections with other PRDC-associated agents [10;11]. In contrast to SIV, PCV2 is considered a primary etiological agent of the PRDC given its immune modulating properties [12]. PCV2 is highly prevalent in PRDC cases and is thought to be a critical agent for development of the PRDC syndrome [5;13]. Vaccines to aid in the control of these viruses are currently on the market but the emergence of novel strains highlights the need for continued vaccine development to adapt to the potential changes in the viral landscape [14-17].

Recombinant DNA technology presents a rapid and powerful method for development of vaccines for emerging pathogens. As some agents are difficult to cultivate *in vitro* or can accumulate mutations which modify their immunogenicity, the use of recombinant antigens can circumvent these problems and potentially lead to more rapid product development. The baculovirus expression vector system (BEVS) represents a versatile and robust system for the expression of recombinant proteins. As the BEVS has been in use for over thirty years, it is a highly developed expression system with a variety of available cloning and expression tools [18]. The maturity, flexibility, and robustness of the BEVS make it an ideal system for development of recombinant vaccines.

Dissertation Organization

This dissertation consists of four chapters centered on the use of the BEVS for the development of vaccines to combat agents associated with the PRDC. Chapter 1 is a review of the relevant literature surrounding the PCV2 and SIV viruses, their associated diseases, and current options for vaccination. In addition, an introduction to the BEVS and its potential uses is provided. Chapters 2 and 3 are manuscripts intended for publication detailing SIV and PCV2 proof of concept vaccines generated using the BEVS and their respective efficacy evaluation in pigs. Chapter 4 provides some general conclusions from the two studies and provides suggestions for future investigation.

For the manuscript in Chapter 2, the author was responsible for the design and evaluation of the baculovirus constructs, generation of antigen and formulation of vaccines. The author served as the Study Director for the vaccination/challenge study and was responsible for all laboratory work associated with the study. In addition, the author was responsible for all data analysis and interpretation of the study results. Eric Vaughn and Cathy Miller served as mentors and reviewers for the document. For the manuscript in Chapter 3, the author was responsible for the conception, design, and evaluation of all baculovirus constructs. In addition, the author generated antigen for the vaccination/challenge study. Greg Haiwick served as the study director for the vaccination/challenge study and directed the laboratory phase of the study. Although the author was not directly involved in the vaccination/challenge study except for the serology evaluation by ELISA, all charts and graphs were prepared by the author from the study data. Statistical analysis of the study data was completed by Brian Fergen. The author was responsible for all other data analysis and interpretation of all results. For both chapters, the author carried out his responsibilities either by himself or through direction of junior scientists.

Literature Review

PCV2 Virus Description and Replication

In the 1990's, a previously undescribed disease in pigs characterized by wasting of the animal and lesions within the lymphoid organs was reported in Canada. Over the following few years, the disease, designated postweaning multisystemic wasting syndrome (PMWS) was reported on other continents around the world. Porcine circovirus type 2 (PCV2), a small single-stranded DNA virus of the family *Circoviridae*, is now recognized as the etiological agent responsible for PMWS. In the ensuing decade and a half, PCV2 has been associated with a large number of disease syndromes and has become one of the most economically important disease agents affecting swine [19].

Comprised of a 1.7kb covalently-closed, circular genome, PCV2 is packaged in an icosahedral non-enveloped capsid, approximately 18nm in diameter [20]. The ambisense genome encodes two replicase proteins, Rep and Rep' (ORF1), a capsid protein, Cap (ORF2), and two accessory proteins thought to be involved in virus-induced apoptosis and transcriptional regulation (ORF3 & ORF4) [21-23]. ORF1 and ORF2 are arranged in opposite orientations flanking the origin of replication with the ORF1 coding sequence on the genomic strand and ORF2 coding sequence on the complementary strand generated during DNA replication (Fig 1) [24]. ORF3 and ORF4 coding sequences are also found on the complementary strand within the ORF1 region [25]. Other putative open reading frames and transcriptional products have been reported but their functions remain unknown [25;26].

The viral genome is replicated via a partially described rolling circle mechanism that is dependent on host polymerases [24;27;28]. The Rep and Rep' gene products contain rolling

circle replication motifs and have been shown to bind the viral DNA in specific regions within a hairpin loop structure located between the ORF1 and ORF2 genes [29-31]. In addition, Rep and Rep' demonstrate nicking/joining activity and are essential for genomic DNA replication [32]. Viral particles are presumably assembled in the nucleus as both the Rep proteins and the Cap protein localize to the nucleus during infection [33-35]. Though it is known that Cap and Rep interact, the exact process and location of viral assembly remain to be determined [36].

Known to bind the glycoaminoglycans (GAGs) heparan sulfate and chondroitin sulfate B, PCV2 can attach to a variety of cell types given the ubiquitous distribution of these GAGs on cellular glycoproteins [37]. PCV2 has been shown to infect hepatocytes, cardiomyocytes, monocytes, and lymphocytes, but since the virus is dependent on host DNA polymerases, viral replication occurs only in actively dividing cells [27;28;38;39]. Replication of PCV2 in hepatocytes and cardiomyocytes generally occurs only in fetuses during the early stages of development when these cells are actively dividing [40]. Infection of monocytic cells does not usually lead to PCV2 replication but rather persistence of the virus. PCV2 has been shown to persist in porcine alveolar macrophages (PAM) *in vitro*, decreasing production of lysosomal hydrogen peroxide and oxygen radicals while modulating genes associated with MHC class I and MHC class II antigen presentation [41-43]. In addition, persistence in monocyte-derived and bone marrow-derived dendritic cells *in vitro* has also been documented [44]. In contrast to PAMs, the immune functions of these cell types do not seem to be affected by the presence of PCV2. It has been proposed that these antigen presenting cells are utilized as vehicles to traffic PCV2 to the lymphoid tissue where the virus may encounter, depending on the host immune status, activated lymphocytes which have been shown to be particularly susceptible targets for PCV2 replication [44-46].

Porcine Circovirus Disease (PCVD)

The most recognized form of PCVD is PMWS, now designated PCV2 systemic disease (PCV2-SD) [47]. PCV2-SD is characterized by stunted growth and wasting in weaned pigs and may include respiratory disease, jaundice and enteritis [48]. Affected pigs may present with enlarged lymph nodes, non-collapsed tan-mottled lungs, white spots on the kidneys, atrophic and/or discolored liver, and catarrhal enteritis. Microscopically, pigs suffering from PCV2-SD display hallmark lesions within the lymphoid tissue consisting of granulomatous inflammation of the lymph nodes, depletion of lymphocytes, and destruction of the lymphoid tissue structure. In addition, high levels of PCV2 may be detected in the serum, lymphoid tissue, and other tissues depending on the severity of the infection. Other forms of PCVD that do not include the hallmark lesions of PCV2-SD, including respiratory disease (PCV-LD) and enteric disease (PCV-ED), also occur in pigs as well as a high number of subclinical infections [47;49]. In addition, PCV2 has been implicated in reproductive disease causing abortions, stillbirths and fetal mummification [50;51]. Experimental reproduction of PCV-SD with PCV2 alone is difficult and typically requires co-factors for full expression of the disease [52;53]. The exact triggers that lead to expression of PCV2-SD are still not well understood but are thought to include co-infection with other pathogens, animal genetics and environmental factors [54-56].

Though PCV2-SD was the first disease to be associated with PCV2, the virus has since been linked to a number of additional disease syndromes affecting pigs including porcine dermatitis and nephropathy syndrome (PDNS) and the PRDC [9;57]. A common thread in these diseases is that they seem to be multifactorial in nature. Porcine dermatitis and nephropathy syndrome is characterized by red-to-purple blotches on the skin that can become crusted and fade over time and is thought to be the consequence of an immune complex disorder. Affected pigs display

little to no fever but can appear depressed and reluctant to move. Although PCV2, PRRSV, and some bacterial pathogens have been associated with this disease, their actual pathogenic contribution remains to be determined [58]. Respiratory disease mediated by the PRDC is characterized by bronchopneumonia and often interstitial pneumonia of the cranioventral portions of the lung with varied histopathology that depends on the pathogens responsible for disease [13]. In addition to PCV2, PRRSV, SIV, *Mycoplasma hyopneumoniae*, *Pasteurella multocida*, and *Actinobacillus pleuropneumoniae* have all been implicated in PRDC-mediated disease [9]. These PRDC- associated etiological agents and the resulting clinical outcomes can overlap and may occur in concert with PCV2-LD and/or PCV2-SD [5;59]. Taken together, PCV2 is associated with a number of diseases in pigs whose etiology and outcomes are complex and potentially overlapping due to their multifactorial nature.

Circulating Strains and Current Treatments

PCV2 strains are categorized into three recognized genotypes (PCV2a, PCV2b, and PCV2c) based on the nucleotide sequence of their ORF2 gene [60;61]. As only three PCV2c strains have been published to date, all associated with PMWS pigs in Denmark, the significance of this genotype is unclear [62]. In contrast, PCV2a stains were those initially isolated from PMWS cases in the 1990s and were historically the most prevalent genotype in the field [63;64]. More recently, a number of studies comparing PCV2 strains isolated from PMWS and non-PMWS pigs indicated that a global shift in genotype from PCV2a to PCV2b had occurred sometime in the early 2000's. The studies also indicated that PCV2b strains were associated with PMWS more frequently than PCV2a strains [62;65-67] . PCV2b is now the predominant circulating genotype and is isolated from cases of PCV2 infection globally.

A number of vaccines to combat PCV2 infection are currently available including Ingelvac CircoFLEX® (Boehringer Ingelheim), Circumvent® (Merck), Fostera PCV® (Zoetis), and Circovac® (Merial). Ingelvac CircoFLEX® and Circumvent® are based on PCV2 ORF2 antigen produced using the baculovirus expression vector system (BEVS) while Fostera PCV® and Circovac® are formulated with inactivated PCV2 virus [68]. These vaccines have been shown to induce neutralizing antibodies, decrease the lymphoid lesions and viremia associated with PCV2 infection, and increase average daily weight gain after challenge [69-72]. The effectiveness of the vaccines has led to their employment globally to help control PCV2 infection. Although the currently circulating strains of PCV2 are predominately of the 2b genotype, the antigens used in the current vaccine formulations are from PCV2a strains. Despite this fact, reports suggest that these PCV2a-based vaccines are efficacious against PCV2b field strains and that cross-protection occurs between PCV2a and PCV2b [73;74].

Antigenic Diversity and the Emergence of Novel Strains

Despite the success of the current vaccines in reducing the impact of PCVD on swine herds, reports continue to surface detailing outbreaks of novel strains in vaccinated herds. In China in 2010, investigators reported novel isolates from pigs suffering from PMWS. Of the 19 isolates reported, three were found to be distinct from the currently recognized genotypes on the basis of nucleotide alignment. In addition, these isolates were not recognized by a conformational PCV2-specific monoclonal antibody that reacted with the other PCV2a and PCV2b isolates reported in the study, suggesting that the nucleotide changes resulted in antigenic modification of the Cap protein. The type strain of this newly proposed genotype was designated BDH and contained a signature mutation, the addition of a lysine at the C-terminus of the Cap protein as a result of mutation of the stop codon [75].

Two years after the initial report from China, a nearly identical isolate associated with vaccine failures was reported in the US, raising concerns that the currently available vaccines may not provide protection against these novel PCV2 isolates [76]. In addition to the findings in the US, a recombinant clone of the Chinese BDH isolate was associated with increased virulence when compared with classical PCV2a and PCV2b strains [77]. Although two later experimental studies suggested that the current PCV2a-based vaccines provide protective efficacy against the BDH-like US isolate, similar isolates have subsequently been be reported in cases of vaccine failure in Korea, Brazil, and most recently Germany [16;78-81]. Alignment of the Cap amino acid sequences of these isolates demonstrates that they all contain the signature amino acid addition at the C-terminus and display >98% amino acid identity. On a genomic level, nucleotide sequence identity between the isolates is >99%, with the BDH isolate and the US isolate containing a single nucleotide substitution in the ORF1 coding region between them. With more data available, the previously proposed new genotype for BDH-like strains, designated PCV2d, has been reclassified as a divergent cluster of the PCV2b genotype designated mPCV2b [82]. The high nucleotide identity between the isolates and their divergence from other PCV2b isolates (96% nucleotide identity on average) suggests a common ancestor that has spread globally despite the constant pressure of vaccination.

The emergence of the mPCV2b strain highlights the continuous evolution of PCV2 viruses in the field which may be accelerated due to the employment of vaccination programs on a global scale. Investigations into the evolution of the PCV2 genome have demonstrated a nucleotide mutation rate of 1.2 - 6.6 x 10^{-3} substitutions per site per year, a rate higher than any reported for a DNA virus and on par with that of single-stranded RNA viruses [83;84]. In addition, reports of recombination between strains *in vitro* and in the field suggest that genetic change may be

further accelerated when multiple strains co-circulate in the same region [85-87]. Taken together, the rapid antigenic drift coupled with the capacity for recombination between strains elevates the potential for the emergence of novel PCV2 strains and underlines the need for continued vaccine development.

Influenza-mediated Respiratory Disease in Swine

Influenza infection in swine is mediated by Influenza A viruses and characterized by sudden onset acute respiratory disease with high morbidity and low mortality [88]. Although historically thought of as seasonal disease similar to influenza infection in humans, recent surveillance has demonstrated that SIV infection can occur year-round [89]. Infected pigs exhibit coughing, sneezing, fever, respiratory distress and inappetence often leading to decreased weight gain. Viral replication leads to necrosis of the bronchial epithelium and release of influenza virus into the airways where it can be detected in nasal swabs and bronchial alveolar lavage (BAL) fluids during infection The lungs of infected pigs present with macroscopic purple-red lung lesions which can range from multifocal to consolidated often in the cranio-ventral lobes. Additionally, microscopic signs within the lungs can include bronchiolitis, interstitial pneumonia and peribronchiolar lymphocytic infiltration [15;90]. Infected pigs typically recover in 4-7 days after the onset of symptoms unless complicated by infection with other disease agents as in PRDC manifestation [4;91].

SIV – Virus Description and Replication

Influenza A viruses are RNA viruses of the family *Orthomyxoviridae* consisting of eight negative-sense single-stranded RNA segments encapsidated in an enveloped pleomorphic particle 100nm in diameter on average [92;93]. The viral envelope contains two types of

glycoprotein peplomers, hemagglutinin (HA) and neuramindase (NA) as well as low pH-active ion channels formed by homotetramers of the matrix 2 (M2) protein [94;95]. The viral capsid is constructed of matrix 1 (M1) protein which is thought to interact with the envelope and binds the eight viral genomic RNAs (vRNAs) [96;97]. The vRNAs are associated with the RNA-dependent RNA polymerase (RdRP) consisting of the polymerase basic proteins 1 & 2 (PB1 and PB2 and the polymerase acidic protein (PA). Each of the eight vRNAs are bound by a RdRP complex via the conserved 5' and 3'ends of the vRNAs forming large loop structures. The free loop portions of the vRNAs are wound around multiple copies of the nucleoprotein (NP) forming tight RNA/protein complexes designated viral ribonucleoprotein complexes (vRNPs) [98]. These vRNPs are the minimal products needed for replication of influenza.

Attachment of Influenza viruses to host epithelial cells is mediated by the HA homotrimers anchored in the viral envelope. HA binds sialic acid residues which are the terminal saccharides on many host surface glycoproteins and glycolipids [99]. Once bound, the virus is internalized by receptor mediated clathrin-dependent or clathrin-independent endocytosis [100]. Acidification of the endosomes during their maturation process triggers a shift in the confirmation of the HA homotrimers, exposing a hydrophobic peptide region termed, the fusion peptide, that is plunged into the host plasma membrane [101]. Upon insertion of the fusion peptide, the HA homotrimer complex folds back on itself, fusing the viral envelope with the host endosomal membrane [102]. Concurrently, the M2 ion channel undergoes structural modification allowing for acidification of the virion resulting in dissociation of the nucleocapsid and release of vRNPs into the cytoplasm [103]. Translocation of the vRNPs into the nucleus is thought to be mediated by nuclear localization signals (NLS) associated with NP [104;105].

Once in the nucleus, the viral genomic RNAs (vRNAs) are replicated and mRNAs are transcribed for expression of the viral gene products. For vRNA replication, complementary RNAs (cRNAs) of each segment are synthesized by the RdRP and subsequently used as template for the generation of progeny vRNAs. In contrast to vRNA generation, transcription of mRNA is initiated by a process called "cap-snatching" where host pre-mRNAs are bound by PB2 and cleaved by PA [106;107]. The resulting 5' Cap and short oligonucleotide sequence is used as a primer for synthesis of mRNAs from vRNAs. mRNAs are polyadenylated independent of host enzymes via slippage of the RdRP that occurs during transcription of the uracil-rich 5' end of the vRNAs [108].

Newly synthesized vRNAs are packaged into vRNPs and interact with the nuclear export protein (NEP) which mediates interaction with M1 and facilitates nuclear export via interaction with host CRM-1 [109-111]. Exported vRNP-M1 complexes are transported to the plasma membrane for budding potentially through vesicular transport mediated by the host microtubule network [112;113]. Budding of the virus occurs from the apical plasma membrane at lipid raft domains, the site of HA and NA migration after maturation in the golgi [114]. Recently, cell-to-cell spread of influenza without budding via intracellular connections has been demonstrated, illuminating a previously unknown pathway for viral dissemination [115].

Antigenic Variation

Influenza A viruses undergo antigenic variation by two methods termed antigenic drift and antigenic shift. Antigenic drift refers to the accumulation of nucleotide mutations over time in viral genomes due to errors introduced by the virus-encoded RdRP. These mutations lead to antigenic diversity within genotypes as those viruses with mutations that give them a competitive

advantage within the host are those that become the most prevalent. Antigenic drift is most readily observed in the hemagglutinin and neuraminidase proteins due to heavy pressure from the host immune system [116]. Antigenic variation by antigenic shift occurs when superinfection of host cells with two or more heterologous viruses produces new viruses that contain a mixture of gene segments from the parent viruses. This mixing of gene segments is referred to as viral reassortment and may be responsible for the emergence of novel influenza genotypes that have caused costly pandemics in humans [117].

Host range for influenza viruses is thought to be determined by the sialic acid-galactose linkages recognized by their respective hemagglutinin proteins. Human influenza A viruses bind sialic acid residues which contain an α2,6 sialic acid-galactose linkage. In contrast, avian influenza A viruses bind sialic acid residues that contain an α2,3 linkage [118]. As pigs express glycans with α2,3 linkages and glycans with α2,6 linkages, they present a potential "mixing vessel" for the emergence of novel influenza genotypes by reassortment [119]. While only a few isolated pig-to-human transmission cases had been reported previously, the 2009 swine flu pandemic underlined the potential of pigs to generate novel and possibly pandemic influenza strains [120-122]. In addition to the potential threat to human health, the capacity of pigs for reassortment has also led to an increased diversity of SIV strains in the swine population in the US.

Circulating Strains and Current Treatments

Despite minimal change for the better part of the 20th century, the landscape of influenza genotypes circulating in swine has become increasingly complex over the last few decades. Initially recognized in 1918 during the Spanish flu pandemic, an H1N1 influenza genotype, now

termed “classical” SIV, circulated in North American swine populations mostly unchanged until 1998 when novel H3N2 viruses emerged [88]. This new viruses were found to be products of reassortment containing H3, N2 and PB1 segments from human H3N2, PB2 and PA segments of avian origin and NP, M, and NS segments from classical SIV [123]. These triple reassortant H3N2 viruses have become endemic in the US swine population and seem to have fostered increased diversity in the genotypes circulating in swine [124;125].

The last decade and a half has produced a large number of novel SIV genotypes that now co-circulate in the swine population. Reassortment between the H3N2 triple-reassortant viruses and classical H1N1 viruses have produced novel H1N1 and H1N2 strains of SIV containing H1 and N1 from classical swine H1N1 viruses and/or N2 from the H3N2 viruses [126]. The residual segments of these novel viruses are similar (swine M, NP, and NS, avian PB2 and PA, and human PB1) and derived from the H3N2 triple-reassortant viruses. As these segments have stabilized, having been found together in a number of novel SIV strains, they have been termed the triple reassortant internal gene (TRIG) constellation [90]. More recently, H1N2 viruses of either completely human origin or reassortant viruses of swine and human origin were detected in Canada [127]. In addition, H1N1 and H1N2 viruses containing H1 and/or N1 from human seasonal strains coupled with the TRIG constellation have also been detected [17]. Furthermore, the emergence of the 2009 H1N1 pandemic virus has led to additional reassortant viruses [128].

The plethora of reassortant viruses coupled with continued antigenic drift has resulted in a multifaceted and dynamic influenza ecological situation in swine [129;130]. Currently, circulating strains can be grouped into one of ten phylogenetic clusters based on their HA segment. H1 SIV strains are categorized as H1α (classical H1N1-like), H1β (triple-reassortant H1N1-like), H1γ (triple-reassortant H1N2-like), H1δ1 and H1δ2 (human H1-like), or H1pdm09

(pandemic H1-like) [17;131]. Similarly, H3 SIV strains are categorized as cluster I, II, III or IV [132;133]. Limited cross-protection between these HA clusters has been demonstrated by multiple researchers. Evaluation of commercial bivalent SIV vaccines containing cluster I H3N2 antigens for efficacy against a H3N2 cluster III challenge virus showed only partial protection with minimal generation of HI titers against the challenge virus and little to no reduction in the number of pigs shedding virus after challenge [134]. Additionally, vaccination of pigs with an H1N1 vaccine provided no protection and actually increased the severity of lung lesions after challenge with an H1N2 isolate [135]. These reports highlight the necessity for multivalent vaccines that must be continually updated to meet the challenge of emerging strains in the field.

The traditional process for the development of SIV vaccines, requiring inactivation of whole virus for formulation, carries a number of disadvantages. Firstly, to produce virus for inactivation, isolates must be adapted to *in vitro* cultivation which typically occurs in embryonated chicken eggs [15]. Requiring a number of passages, the adaptation process and subsequent manufacturing steps can lead to mutations that may affect the immunogenicity of the resulting product. In addition, some isolates may not propagate to sufficient titers in *in vitro* systems or worse, not replicate at all [136]. Compounding these issues, SIV vaccines must be multivalent to ensure broad protection against the complex landscape of circulating strains as demonstrated by two of the currently licensed vaccines, FluSure XP® (Zoetis) and MaxiVac Excell® 5.0 (Merck), which contain four and five strains respectively [137;138]. In addition to the challenges associated with *in vitro* cultivation, licensure of SIV vaccines requires the demonstration of safety and efficacy through multiple animal studies. For multivalent vaccines, animal studies become more complex with the need to demonstrate the absence of immunological interference between the included antigens. Taken together, the disadvantages

of inactivated whole virus vaccines can result in inflated product costs and lengthy development timelines [139]. Although these traditional SIV vaccines have been shown to be effective in protecting pigs against SIV, the swiftly changing landscape of influenza in swine demands a more rapid and flexible platform for vaccine development.

Introduction to the BEVS

First described in 1983, the BEVS is one of the most developed and versatile recombinant protein expression systems in use today [18;140-142]. Forming a bridge between the highly utilized *E. coli* expression systems and the more specialized mammalian expression systems, such as transient or stable expression in CHO cells, the BEVS combines ease of construct development with the advantages of eukaryotic protein folding systems and post-translational modifications [143]. The central concept of the BEVS is protein expression driven from promoters dispensable for viral replication during infection of insect cells [144]. As baculoviruses shutdown translation of host gene products, high levels of recombinant protein can be achieved during the infection process [145]. The BEVS has been used to produce a variety of protein products including soluble subunit proteins, glycoproteins, virus-like particles (VLPs), and multiprotein complexes [146-149]. In addition, the BEVS has proven its worth in commercial production applications as vaccines registered for human and veterinary use are currently on the market [68;150]. Although the majority of experimental and commercial utilization of the BEVS has focused on a single baculovirus, *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV), lesser utilized expression systems have been developed around other members of the large baculovirus family [151].

Baculovirus Biology

Baculoviruses are a large family of enveloped DNA viruses that infect the larval stage of insects of the order *Lepidoptera* (moths and butterflies), *Hymenoptera* (wasps, bees, and ants), and *Diptera* (flies) [152]. These viruses consist of a circular double-stranded DNA genome of 80 – 180 kb packaged into an enveloped rod-shaped viral particle 30 – 60 nm in diameter and 250 – 300nm in length [142]. Like other large DNA viruses, baculoviruses replicate in the nucleus and encode a substantial number of proteins that coordinate transcription of the viral genes, carry out replication of the viral genome, and interfere with cellular process to gain a competitive advantage in the hostile environment of the insect cell [145;153-155].

Upon replication, two distinct phenotypes of viral progeny are generated; the occlusion-derived virion (ODV) and the budded virion (BV) [156]. The nucleocapsids of both virion phenotypes are assembled in the nucleus but become enveloped via different pathways. Although the mechanism is still not completely understood, ODVs are known to be enveloped in the nucleus presumably by microvesicles that bud from the nuclear membrane [157;158]. The enveloped ODVs are further processed by packaging into crystalline structures called occlusion bodies made of polyhedrin or granulin protein in the nucleus [159]. In contrast, the BV nucleocapsids are exported from the nucleus and enveloped at the plasma membrane similar to other enveloped viruses.

The BVs and ODVs carry out two distinct functions during the baculovirus lifecycle. ODVs promote persistence of the virus in the environment and initiation of infection of the host while the BVs spread the infection throughout the tissues, eventually leading to death of the host. Due to their encapsidation into the polyhedrin-based or granulin-based occlusion bodies, ODVs are

quite stable in the environment. Once ingested by the insect host, the occlusion bodies begin to break down due to the high pH of the insect midgut [160]. Upon dissolution of the occlusion bodies, ODVs are released into the midgut where they attach to the epithelial cells, initiating the infection in the host. The BVs generated from the initial infection spread from the basal side of the epithelial cells, disseminating the infection into the surrounding tissues. As the infection rapidly progresses, BVs and ODVs are generated leading to death of the host. For some baculoviruses, the host is liquefied via chitinase, which breaks down the insect exoskeleton, further facilitating release of the ODVs into the environment [156].

Expression of baculovirus genes is regulated in three distinct phases designated, early, late, and very late. The early phase genes have been shown to be transcribed by host RNA polymerases and are involved in replication of the DNA genome, control of host processes, and regulation of the later phase gene products [161]. As the infection transitions from early to late phase, genomic DNA replication is concluded and translation of host gene products is inhibited by viral antagonists. The late genes are associated with production of viral structural proteins including the nucleocapsid proteins and the major BV surface glycoprotein gp64. During this phase, BVs are heavily manufactured as nucleocapsids are transported from the nucleus and enveloped at the plasma membrane. In the very late phase, gene products associated with ODV and occlusion body formation, including the *polh* (polyhedrin) and *p10* genes, are expressed [151]. These genes are highly up-regulated during the very late phase of infection leading to high concentrations of the proteins in the cell. As ODV and occlusion body formation are dispensable for baculovirus replication in cell culture, the *polh* and *p10* promoters have been exploited to express foreign gene products forming the basis for the AcMNPV BEVS [162].

Generation of Recombinant Baculovirus

To generate recombinant baculovirus (rBV), the coding sequences for target recombinant proteins are inserted into the baculovirus genomic DNA by homologous recombination or transposition via two general methods. Originally described in 1983, co-transfection of insect cells with baculovirus DNA and a plasmid containing the gene of interest flanked by baculovirus genomic DNA sequences continues to be in use [163]. Today, purified baculovirus DNA is available from multiple manufacturers and may contain inserted or deleted genes aimed at improving recombinant protein production [164;165] . Although early processes for generating recombinant baculovirus by this method required multiple rounds of screening and plaque purification, currently available baculovirus DNA backbones are linearized, resulting in a lethal deletion of ORF 1629 that increases recombination efficiency to near 100% [166].

The second method for insertion of foreign coding sequences involves the use of baculovirus genomic DNA that has been incorporated into a bacterial artificial chromosome (BAC). The baculovirus BAC, termed a "bacmid" is modified by homologous recombination or the use of transposon elements in *E. coli*. The modified baculovirus DNA is purified and subsequently transfected into insect cells to produce recombinant baculovirus. The advantage of these systems is that all progeny virus generated from transfection contain the recombinant bacmid DNA, removing the need for screening and plaque purification. In addition, modifications to the baculovirus backbone can be readily produced in the *E. coli* system [167;168]. Although the bacmid systems are desirable for their efficiency and ease of modification, the fact that recombinant baculovirus prepared with these systems can contain antibiotic resistance markers or remnants of transposable elements makes them somewhat unsuitable as vectors for vaccine development. In addition, recombinant baculovirus generated using bacmid methods have been

shown to spontaneously delete the inserted recombinant DNA from their genomes upon serial passage, making them particularly risky for commercial production applications [169].

Insect Cell Lines and Recombinant Protein Expression

Although there are a wide variety of insect cell lines that can be used for the production of recombinant proteins using the BEVS, the most commonly used cell lines are derived from lepidopteran insects [170]. The cell line IPLB-Sf21-AE, commonly referred to as Sf21, was isolated from the ovaries of fall army worm (*Spodoptera frugiperda*) pupa [171]. A subclone of Sf21, designated Sf9, is also commonly employed for protein production using the BEVS. A third cell line isolated from cabbage looper (*Trichoplusia ni*), is designated BTI-Tn-5B1-4 (Tn-5) and is sold commercially as High Five™ (Life Technologies) [172]. The success of these three cell lines is a product of their ability to propagate in suspension culture in serum-free media, conditions that highly suitable for commercial production [151].

Transcription and subsequent expression of recombinant protein in the BEVS is typically driven by the *polh* promoter though other promoters can be used in multi-gene expression scenarios or when expression at earlier times during infection is needed. The *p10* promoter is a very late promoter that is often used in concert with *polh* to drive expression of multiple gene targets simultaneously [173;174]. Given the lytic in nature of the rBV infection, the timing with which recombinant proteins are harvested can be quite important and depend specifically on the protein of interest. For proteins that are particularly susceptible to proteolytic degradation, peak expression can be moved to the late phase of the infection cycle using the *p6.9* promoter and harvested prior to the build-up of cellular contents that occurs during the lytic portion of the infection curve [175].

An advantage of the BEVS over prokaryotic expression systems is the ability of insect cells to perform eukaryotic post-translational modifications including N-linked glycosylation. Insect produced N-linked glycans are less complex than their mammalian counterparts, consisting of oligo-mannose residues attached to the N-acetylglucosamine backbone. In contrast, mammalian N-linked glycans contain more diverse saccharide residues and typically terminate in sialic acid (Fig 2) [176]. The difference in N-linked glycans is a significant problem for BEVS-produced recombinant proteins designed for biotherapeutic use as biological function may be negatively impacted. In addition, the foreign N-linked glycans may increase the immunogenicity of BEVS-produced biotherapeutic proteins [177]. To deal with this problem, modified baculovirus backbones and recombinant insect cell lines have been developed that provide additional enzymes for more "mammalian-like" glycan processing in insect cells [178;179]. With regard to vaccine development, the difference in N-linked glycans may be an advantage given the potential for increased immunogenicity. In addition, the more simplified glycans may reveal epitopes that may have been shielded by more complex glycans in the native protein.

Versatility of the BEVS

Proteins expressed from the BEVS have been used for a wide range of applications including biochemical and structural studies, biotherapeutics, vaccine development, and more recently gene delivery applications. The preparation of recombinant proteins for crystallography studies is a frequent use for the BEVS and in fact has become the most common method for producing crystallography-quality recombinant proteins [143]. A diverse range of proteins have been successfully crystallized from BEVS preparations including receptors, cytokines, enzymes, and surface glycoproteins [146;180-182]. In addition to proteins for structural studies, the BEVS is often used to produce biotherapeutics [183]. The development of transformed insect cell lines

and modified baculovirus vectors that encode addition enzymes for more human-like glycoprotein processing has helped generate less reactive and more functional recombinant biotherapeutics [178;184]. In terms of vaccine development, viral glycoproteins and structural proteins have been an intense area of focus as the BEVS is capable of producing both of these target types with proper post-translational modifications and oligomerization.

Immunogenic viral glycoproteins have been produced with the BEVS in both membrane-associated and secreted forms for a number of viral targets. Generation of membrane-associated glycoproteins requires targeting of glycoproteins to the insect cell plasma membrane which can be accomplished using native coding sequences. The resulting membrane-anchored glycoproteins, which are extracted using detergents and purified by chromatography methods, have been shown to be similar to the native viral proteins in terms of antigenicity and may elicit neutralizing antibody responses as has been demonstrated for glycoproteins from both RNA and DNA viruses [185-189]. In fact, a commercial human influenza vaccine, FluBlok®, is produced using these methods [190]. In order to get around the detergent extraction protocols typically needed for purification of the resulting membrane-associated glycoproteins, some researchers have expressed these targets as secreted proteins, removing the transmembrane domain and in some cases adding additional protein sequences such as immunoglobulin Fc domains (IgG Fc) or the T4 fibritin domain to promote stability and oligomerization of the secreted target [191-193]. Alternatively, glycoproteins can be efficiently displayed in the baculovirus envelope by fusion with the signal peptide and c-terminal domain of the major baculovirus envelope glycoprotein gp64 [194]. This allows for the use of the baculovirus particle, studded with the glycoprotein of interest, as the immunizing antigen which has been demonstrated to elicit robust humoral and cellular immune responses and provide protective efficacy against the parent virus [195].

Production of enveloped and non-enveloped VLPs using the BEVS has also been described. A large volume of work has been published focusing on the production of influenza VLPs for use as potential vaccines [196]. Utilizing influenza M1 or structural proteins from other viruses enveloped VLPs containing neuraminidase and/or HA glycoproteins, as well as M2, have been produced. These influenza VLPs have been shown to generate both humoral and cell-mediated responses and provide protective efficacy in a number of small animal species [197-200]. Similarly, construction of enveloped VLPs using the BEVS has also been described for a number of other viruses including Rift Valley fever virus, SARS coronavirus, and human immunodeficiency virus [201-203]. In addition to the production of enveloped VLPs, the BEVS provides an efficient platform for the assembly of non-enveloped VLPs. Both single protein VLPs, such as those produced by the infectious bursal disease virus VP2, and multiple protein VLPs, such as the complex capsid of bluetongue virus have been expressed successfully [204;205]. In fact, BEVS produced VLPs are the basis for commercial vaccines for human papillomavirus and PCV2 [150;206].

Conclusions

PCV2 and SIV are important viral pathogens associated with the PRDC that continue to cause economic losses for the swine industry despite the availability of efficacious vaccines. Both of these viruses display the ability to modify their major antigenic determinants through antigenic drift, and in the case of influenza, antigenic shift, highlighting the need for continued vaccine development. With the ability to efficiently generate a variety of recombinant glycoproteins and VLPs, the BEVS is a valuable tool for the development of vaccines against these viruses.

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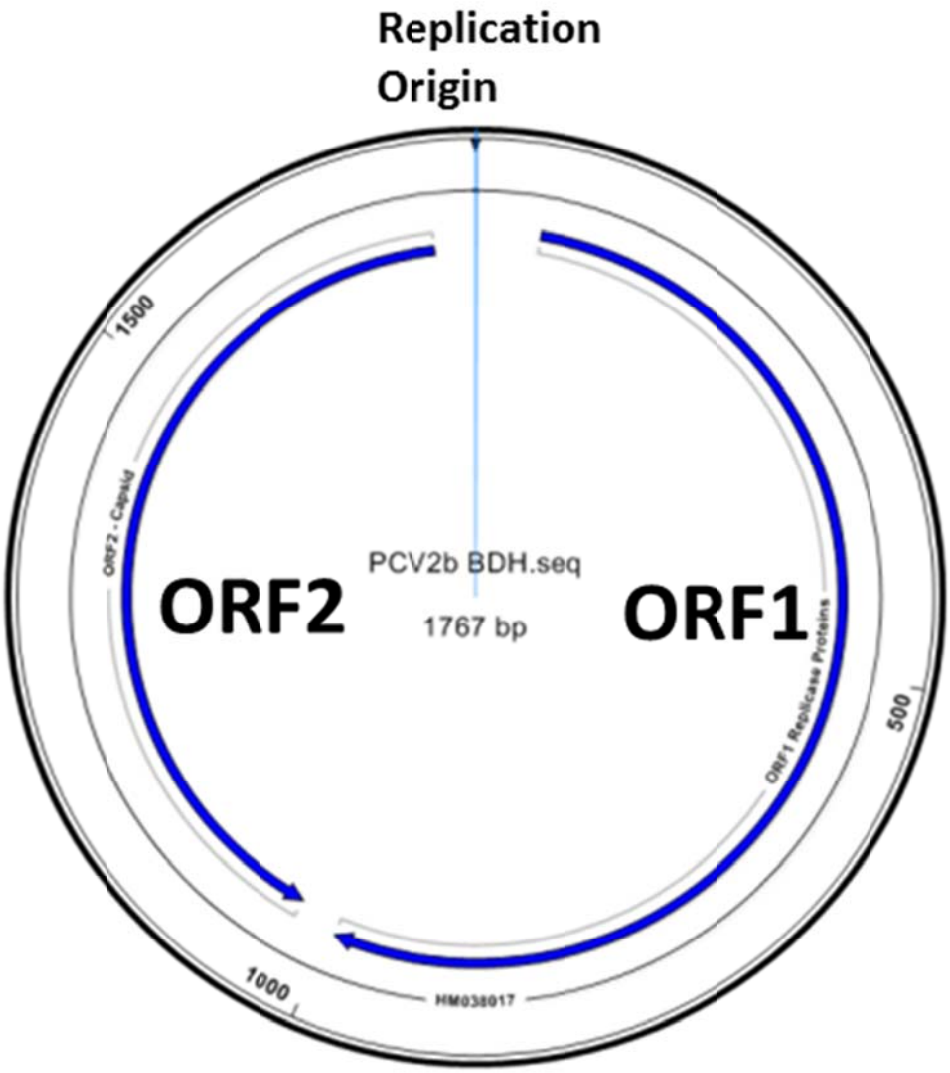

Fig 1. Genomic organization of PCV2. ORF1 is encoded on the genomic strand while ORF2 is encoded on the complementary strand. ORF3 and ORF4 are encoded on the complementary strand in the ORF1 region.

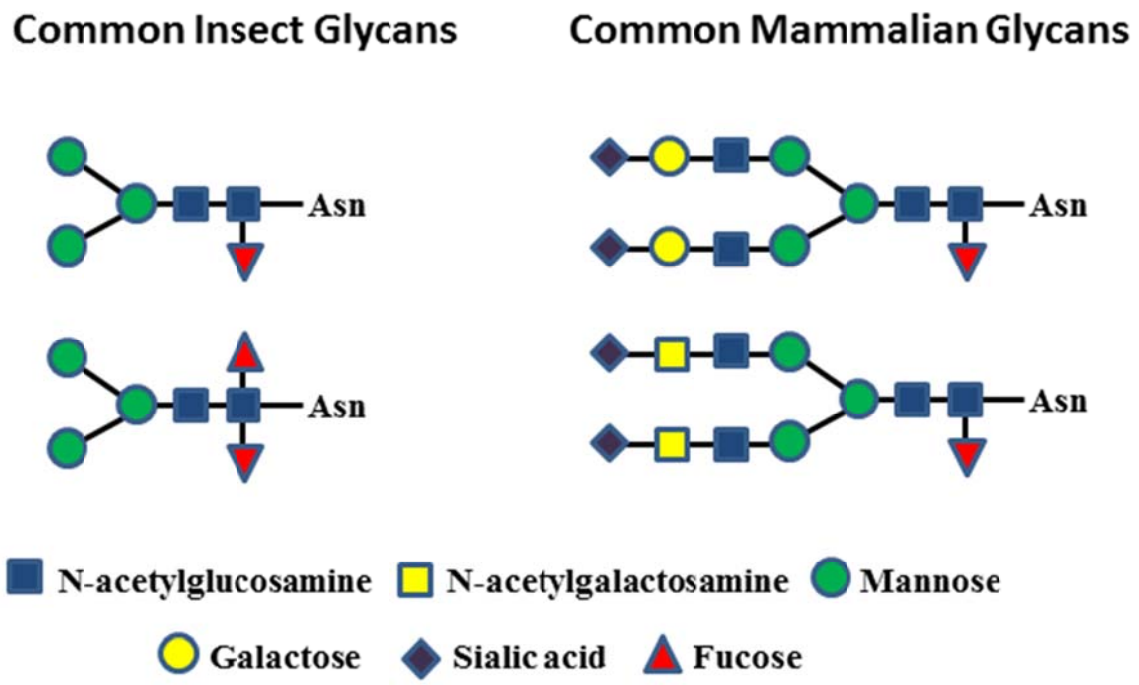

Fig 2. Common insect and mammalian N-glycans. Figure adapted from van Oers et. al. 2015.

CHAPTER 2: PARTICLE AND SUBUNIT-BASED HEMAGGLUTININ VACCINES PROVIDE PROTECTIVE EFFICACY AGAINST H1N1 INFLUENZA IN PIGS

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Abstract

The increasing diversity of influenza strains circulating in swine herds escalates the potential for the emergence of novel pandemic viruses and highlights the need for swift development of new vaccines. Baculovirus has proven to be a flexible platform for the generation of recombinant forms of hemagglutinin (HA) including subunit, VLP-displayed, and baculovirus-displayed antigens. These presentations have been shown to be efficacious in mouse, chicken, and ferret models but little is known about their immunogenicity in pigs. To assess the utility of these HA presentations in swine, Baculovirus constructs expressing HA fused to swine IgG2a Fc, displayed in a FeLV gag VLP, and displayed in the baculoviral envelope were generated. Vaccines formulated with these antigens were administered to groups of pigs who were

subsequently challenged with H1α cluster H1N1 swine influenza virus (SIV) A/Swine/Indiana/1726/88. Our results demonstrate that vaccination with any of these three vaccines elicits robust hemagglutinin inhibition titers in the serum and decreased the severity of SIV-associated lung lesions after challenge when compared to placebo-vaccinated controls. In addition, the number of pigs with virus detected in the lungs and nasal passages was reduced. Taken together, the results demonstrate that these recombinant approaches expressed with the baculovirus expression vector system may be viable options for development of SIV vaccines for swine.

Introduction

Influenza A viruses (IAV) are enveloped, segmented, negative-sense RNA viruses that infect birds and a variety of mammals including humans and swine. A significant problem for swine producers, swine influenza virus (SIV) infection ranks as one of the top three respiratory challenges in breeding, nursery, and finishing operations where estimated additional costs associated with the disease can reach $10.31 per pig to market [1;2;2;3]. In addition to its economic impact on the swine industry, SIV poses a significant threat to human health due to the susceptibility of pigs to both avian and human influenza virus strains. As demonstrated by the 2009 outbreak of pandemic influenza strain A/H1N1/09, pigs can serve as “mixing vessels” with the capacity to generate novel, potentially pandemic, influenza stains via reassortment [4].

Although a single SIV subtype, “classical” H1N1, predominated for decades in North American swine populations, the current landscape of swine influenza viruses is much more heterogeneous. With the emergence of triple reassortant H3N2 SIV at the turn of the century, reassortant viruses containing human, swine and avian gene segments have become common in

US swine herds [5]. The resulting antigenic variation has led to minimal cross protection among currently circulating strains, presenting a significant challenge in controlling SIV infection. Although vaccines are available to combat SIV infection, these products contain only the major circulating strains as they are mostly based on inactivated formulations of field isolates that have been adapted to *in vitro* cultivation [6]. The adaptation of field strains to *in vitro* cultivation carries two inherent disadvantages. First, some field strains may not propagate to acceptable titers by standard *in vitro* culture methods, including embryonated chicken eggs and cell culture. Second, the *in vitro* adaptation process can introduce mutations that may negatively impact vaccine efficacy of the final product. These disadvantages coupled with the strict USDA licensing requirements for influenza vaccines can lead to extended development timelines of up to 5 years, hampering the response time to emerging strains [5]. In order to more quickly respond to outbreaks of new SIV strains not controlled by the currently licensed vaccines, a more rapid and flexible solution is needed.

Recombinant vaccines based on the major influenza antigens present a favorable alternative to traditional inactivated virus vaccines. By tapping into established expression platforms, recombinant antigens can be produced quickly with the flexibility for exchange of antigens as new strains emerge. The baculovirus expression vector system (BEVS) is an established expression platform that is currently in use for human and veterinary vaccines [7;8]. A large number of studies have been published detailing the efficacy of experimental recombinant influenza vaccines prepared using the BEVS. Subunit approaches, involving expression of hemagglutinin (HA) and subsequent purification by anion exchange and/or lentil lectin chromatography have been reported [9-11]. Enveloped virus-like particle (VLP)-based approaches displaying HA and/or neuraminidase (NA) via particles comprised of influenza or

other virus structural proteins have also been reported [9;12-14]. In addition, published reports demonstrate that the baculovirus itself can be utilized in envelope-display and/or transduction based approaches [15-17]. Although the published data suggest that these approaches are promising candidates for new influenza vaccines, the majority of the efficacy data has been generated in mice or birds with minimal efficacy data available in pigs [18].

In order to address the scarcity of swine-based efficacy data and determine the utility of these recombinant approaches for the development of influenza vaccines for swine, several baculovirus constructs expressing various iterations of recombinant HA were generated in this study. Recombinant HA was designed as a fusion protein with a swine immunoglobulin Fc domain (H1-2aFc), modified for insertion into the baculovirus envelope (H1-BD), or expressed in tandem with feline leukemia virus (FeLV) Gag (H1-Fgag). The recombinant HA antigens expressed from these constructs were evaluated for efficacy against a heterologous, classical SIV H1N1 challenge in pigs.

Materials and Methods

Cells and virus

Semi-adherent Sf9 insect cells were maintained in TNM-FH medium (BD Biosciences, San Jose, CA) at 28^{o}C in tissue culture flasks. Suspension SF+ insect cells were maintained in SF900III SFM (Life Technologies, Grand Island, NY) at 28^{o}C in spinner flasks. MDCK cells were maintained in EMEM (Sigma Aldrich, St. Louis, MO) supplemented with 10% fetal bovine serum at 37^{o}C with 5% CO_2. H1N1 SIV A/Swine/Indiana/1726/88 was maintained in specific pathogen-free (SPF) chicken eggs.

Plasmids

Baculovirus constructs were prepared using H1 hemagglutinin (H1DB) from swine influenza virus strain NVSL 96-37181 (H1α cluster) in plasmid pVL1393-H1DB. For H1-2aFc, the H1DB coding sequence was amplified by PCR with primers designed to remove the signal sequence, transmembrane domain and C-terminal tail. The truncated H1DB coding sequence was cloned into pVAX1#4-IgG2aFc in frame with a mouse κ-light chain signal sequence on the 5' end and a swine IgG2a Fc domain, including a 6X GGS linker, on the 3' end. The entire coding sequence was then excised via BamHI/NotI restriction sites and cloned into pVL1393 resulting in pVL1393-H1-2aFc. For H1-BD, a truncated H1DB coding sequence was amplified similar to that of H1-2aFc but without BamHI restriction sites. Synthetic ssDNA fragments encoding baculovirus gp64 signal peptide and C-terminal tail were prepared (Integrated DNA Technologies, Iowa City, IA) and attached to the truncated H1 coding sequence on the 5' and 3' ends respectively by overlap-extension PCR (OE-PCR). The fused coding sequence was cloned into pVL1393 via BamHI and NotI restriction sites resulting in pVL1393-H1-BD. For H1-Fgag, full length H1DB was excised from pVL1393-H1DB via BamHI and NotI restriction sites and cloned into the multiple cloning site 1 (MCS1) of plasmid pORB-MCS1-WSSV IRES-FeLV gag resulting in pORB-H1DB-WSSV IRES-FeLV gag (Fig 1).

Generation of recombinant baculovirus

pVL1393-H1-2aFc, pVL1393-H1-BD, and pORB-H1DB-sIRES-FeLV gag were used to generate recombinant baculovirus BacDB-H1-2aFc, BacDB-H1-BD, and BacFBU-H1-Fgag respectively by co-transfection of Sf9 cells with linearized Diamond Bac (BacDB) or FlashBAC ULTRA (BacFBU) baculovirus DNA. Recombinant baculoviruses were amplified on Sf9 cells and harvested by centrifugation at 1000g for 5 min. Harvest supernatants were further processed

by 0.2μm filtration and stored at 4°C. Amplified baculoviral stocks were titered on Sf9 cells by a fluorescent antibody infectious dose 50 ($FAID_{50}$) method. Briefly, baculoviral stocks were 10-fold serially diluted in cell medium and Sf9 cell layers in 96-well plates were infected with each dilution set, 10 wells per dilution, on duplicate plates. The plates were incubated at 28°C for 5 days, after which, the media was discarded and the cell layers were fixed with 50:50 acetone/methanol. Baculovirus-infected wells were detected by an indirect fluorescent antibody (IFA) test using anti-baculovirus gp64 monoclonal antibody AcVI (eBioscience, San Diego, CA). Infected/non-infected wells were determined for each dilution and the viral titer in $FAID_{50}$/mL was calculated using the Reed-Muench method.

Evaluation of recombinant HA constructs

Recombinant baculovirus was used to infect SF+ cell cultures in spinner flasks at 28°C with agitation at 100rpm. BacDB-H1-2aFc cultures were incubated for 3 days while BacDB-H1-BD and BacFBU-H1-Fgag cultures were incubated for 5 days. At harvest, culture supernatants were centrifuged at 1000g for 5 min to pellet the remaining cells. The clarified supernatants were further processed by 0.2μm filtration to remove any remaining cellular debris. Final harvests were used to evaluate H1 expression from each construct.

For BacDB-H1-2aFc, harvests were directly assessed by SDS-PAGE and Western blot using rabbit anti-H1 polyclonal serum with peroxidase-conjugated goat anti-rabbit IgG as a secondary antibody or goat anti-swine IgG conjugated with peroxidase alone (Jackson ImmunoResearch, West Grove, PA). In addition, harvests were processed via protein A affinity chromatography and SDS-PAGE to confirm fusion of H1 with the IgG2a Fc domain.

For BacDB-H1-BD and BacFBU-H1-Fgag, harvest samples were pelleted by ultracentrifugation at 100,000 x g for 2 hrs at 4^{o}C to pellet large molecular weight species. The resulting supernatants were removed and pellets were resuspended in Tris-buffered saline (TBS, 20mM Tris, 150mM NaCl, pH 7.5). Resuspended pellets were separated on discontinuous sucrose gradients (10% - 60% sucrose in 10% steps) by ultracentrifugation at 100,000 x g for 2 hrs at 4^{o}C. Gradients were fractionated from the top down and analyzed by SDS-PAGE and Western blot utilizing the previously described anti-H1 serum and mouse monoclonal antibody against FeLV gag p27 (BaculoFBU-H1-Fgag only) coupled with peroxidase conjugated goat anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA). Fractions containing H1 (BacDB-H1-BD) or H1 and FeLV gag (BacFBU-H1-Fgag) were pooled and dialyzed against TBS, pH 7.5 to remove sucrose. Dialyzed pooled fractions were concentrated and analyzed by negative stain transmission electron microscopy (TEM) at the National Animal Disease Center (NADC) (Ames, IA).

Formulation and administration of vaccines

Test antigens were generated in SF+ cells by infection with the respective recombinant baculovirus as described in section 2.4. H1-2aFc was purified from harvest fluids by protein A affinity chromatography and dialyzed into TBS, pH 7.5 prior to formulation. H1-2aFc vaccine was formulated with 200μg per dose of purified H1-2aFc protein. For H1-BD and H1-Fgag, each harvest was tested for hemagglutination activity using a standard hemagglutination assay. Briefly, harvests were 2-fold serially diluted in PBS, and incubated with an equal volume of 0.5% turkey red blood cells (tRBCs) in round bottom wells at room temperature (30 - 60 min). The last dilution to form a lattice after incubation was reported as the HA titer. H1-BD and H1-Fgag vaccines were formulated to contain 2048 hemagglutination units (HAU) per dose. The placebo

vaccine was formulated with 50% TBS and 50% Sf900III media. All vaccines were formulated with 20% Emulsigen D as adjuvant. Vaccines were administered intramuscularly (2mL per dose) in alternating neck regions, midway between the base of the ear and the point of the shoulder.

Animals, housing and experimental design

Forty four pigs, seronegative for PRRSV and SIV, and approximately three weeks of age, were blocked by weight and randomly assigned to 5 treatment groups. Pigs were comingled between pens and rooms until day of challenge when the control group (Group 5) was moved to a separate room for the duration of the challenge period. Pigs in groups 1 - 4 were vaccinated on Day 0 and Day 21. Pigs in Groups 1 - 4 were challenged on Day 35 and all pigs were euthanized for necropsy on Day 40. Animals were handled in accordance with the Veterinary Resources, Inc. (VRI) Animal Care and Use Committee and the relevant VRI Animal Care and Use Standard Operation Procedures (VRI, Cambridge, IA).

Clinical observations and sample collection

The general health status of all pigs was observed daily for the duration of the study. Serum samples were collected prior to each vaccination and prior to challenge. Nasal swabs and rectal temperatures were collected on the day of challenge and daily during the challenge period. Weights were collected prior to the first vaccination, at challenge, and at necropsy. Serum samples and nasal swabs were stored at -50^{o}C or colder until testing.

Challenge and necropsy

Pigs were anesthetized just prior to challenge with an IM injection of a combination product containing ketamine, xylazine and tiletamine. Once anesthetized, 2.0 mL of SIV H1N1

A/Swine/Indiana/1726/88 was administered intratracheally to each pig at $7.78x10^6$ 50% egg infectious dose 50 (EID_{50}) per mL using a laryngoscope, syringe and catheter.

On Day 40, serum samples, nasal swabs, rectal temperatures, and body weights were taken. After sample collection, all pigs were euthanized with pentobarbital/phenytoin according to AVMA guidelines and necropsied. At necropsy, the lungs of each pig were removed, observed and palpated for gross lung lesions. Lungs of necropsied animals were scored for percent lung consolidation related to SIV infection. A general description of lung pathology observed and the percentage of pathology in each lung lobe was recorded. The percentage score for each lobe was multiplied by the ratio of the respective lobe weight to the total weight of the lung. The weight-adjusted percentage scores were summed to give the final lung score for each lung[19]. After the lungs were evaluated for pathology, 40mL of PBS was used to flush the lungs to collect a bronchial alveolar lavage (BAL) fluid sample from each set of lungs. BAL fluid samples were stored at -50^{o}C or colder until testing.

Serology

Serum samples were heat-inactivated at 56^{o}C for 30 min then treated with a 20% kaolin solution and absorbed with 0.5% tRBCs prior to testing to remove any agglutinating agents present in the samples. Hemagglutination inhibition (HI) antibodies to SIV were detected by a standard HI assay. Briefly, treated serum samples were 2-fold serially diluted in PBS in duplicate and allowed to pre- incubate with H1N1 SIV A/Swine/Indiana/1726/88 of known hemagglutination titer in round bottom wells. The serum/virus mixture was then supplemented with 0.5% tRBCs and allowed to settle at room temperature (30 – 60 min). The reciprocal of the last dilution to inhibit lattice formation was reported as the HI titer.

Detection of SIV in nasal swabs and BAL fluids

Nasal swabs were thawed, 0.2μm filtered, and used to inoculate duplicate wells of MDCK cells. The samples were allowed to adsorb to the MDCK cells for 1 hour then supplemented with serum-free growth media containing 2 units/mL porcine trypsin. The inoculated MDCK cultures were incubated at 37^{o}C with CO_2 at 5% for 5 – 7 days. The culture supernatants were then harvested with duplicate samples pooled. BAL fluid samples were thawed, 0.2μm filtered, and immediately injected into 9 - 11 day embryonated chicken eggs in duplicate. The eggs were incubated at 37^{o}C for 3 days to allow propagation of virus present. After three days, the allantoic fluid was harvested and pooled from duplicate eggs. Each culture supernatant or allantoic fluid pool was tested in duplicate for the presence of SIV by a standard HA assay. Briefly, an equal volume of 0.5% tRBCs were added to the duplicate samples in round bottom wells and allowed to settle at room temperature. Formation of a lattice after incubation was recorded as a SIV-positive result.

Statistics

All statistical analysis was done using GraphPad Prism 6.05 (GraphPad Software, Inc, La Jolla, CA). Weight gain and log_2-transformed HI titer differences between groups were evaluated by one-way ANOVA with subsequent pairwise comparisons done using Tukey's multiple comparisons test. For lung scores, differences between groups were assessed using a Kruskal-Wallace test with pairwise comparisons done using Dunn's multiple comparisons test. For all analysis, a P-value of less than or equal to 0.05 was considered significant.

Results

Evaluation of Baculovirus-expressed H1 constructs

With the goal of evaluating baculovirus-expressed recombinant HA preparations for efficacy in swine, three baculovirus constructs were prepared and their HA products were assessed by multiple methods. To confirm expression of H1-2aFc, supernatants from BacDB-H1-2aFc-infected SF+ cells at 3 days post-infection were evaluated by SDS-PAGE and Western blot. The results revealed the presence of an approximately 110kDa protein detected by both anti-H1 and anti-swine IgG antibodies suggesting that the H1-2aFc fusion protein was produced as expected (Fig. 2A). Subsequent extraction of the harvest supernatant with protein A agarose beads produced a similar sized band further confirming the presence of the H1-2aFc fusion protein (Fig 2B).

To determine if H1-BD and H1-Fgag proteins were associated with large molecular weight particles, supernatants from BacDB-H1-BD or BacFBU-H1-Fgag-infected SF+ cells at 5 days post-infection were processed by ultracentrifugation. The resulting pellets were resuspended and separated on sucrose gradients. Subsequently, fractions were analyzed for H1 (BacDB-H1-BD) or H1 and FeLV gag (BacFBU-H1-Fgag) by SDS-PAGE and Western blot (Fig. 2C&D). The majority of the hemagglutinin expressed from BacDB-H1-BD and BacFBU-H1-Fgag was detected in the mid (4-9) or lower (9-12) fractions of the gradients respectively. For the BacFBU-H1-Fgag gradient, FeLV Gag was also detected in fractions 9-12 suggesting that the H1 and FeLV Gag proteins expressed by this construct were associated with particles of similar size and density. Particle morphology for each construct was visualized by TEM analysis of pooled fractions containing H1 (BacDB-H1-BD) or H1 and FeLV Gag (BacFBU-H1-Fgag). The

BacDB-H1-BD pool contained rod-shaped enveloped particles typical of baculovirus [20]. In contrast, the BacFBU-H1-Fgag pool contained circular to irregular shaped particles consistent with FeLV gag particles (Fig. 2E) [21].

The functionality of the recombinant H1 proteins was assessed by a standard hemagglutination assay. Both H1-BD and H1-Fgag demonstrated hemagglutination titers greater than the influenza virus control. In contrast, H1-2aFc did not demonstrate hemagglutination activity, even when purified protein was used in the assay (Fig. 3). As H1-2aFc is a subunit protein and not expected to have the multivalency of HAs available on a viral particle or VLP, the lack of hemagglutination activity was not surprising. Taken together, the data demonstrate that the baculovirus constructs expressed the expected HA antigens and were suitable for evaluation of efficacy in pigs.

Recombinant H1 vaccines generate SIV H1N1-specific HI titers in pigs

In order to assess the immunogenicity of the H1-2aFc, H1-BD, and H1-Fgag antigens, vaccines were formulated and administered to groups of 10 pigs as described in Table 1. Group 1 was administered a placebo vaccine as a control, while groups 2, 3, and 4 were given vaccines formulated with H1-2aFc, H1-BD, and H1-Fgag respectively. A fifth group of 4 untreated pigs was included to control for the health of the animals during the study period. To determine if the vaccines elicited SIV H1N1-specific antibody responses, serum samples were evaluated by a HI assay. Twenty-one days after the initial immunization, HI titers against SIV H1N1 (A/Swine/Indiana/1726/88) were detected in the serum of some pigs in the H1-2aFc, H1-BD and H1-Fgag vaccinated groups (Fig 4). When serum samples at 14 days post the second vaccination were evaluated, HI titers were detected in the serum of all pigs vaccinated with the H1-2aFc, H1-

BD, or H1-Fgag experimental vaccines. In contrast to the groups vaccinated with the experimental vaccines, the placebo and control groups did not develop detectable HI titers prior to challenge (Fig 4). When HI titers were compared across the experimental vaccine groups, the mean HI titer of the H1-2aFc group was found to be significantly increased over the H1-BD and H1-Fgag vaccinated groups. These serology data suggest that the experimental recombinant H1 vaccines elicit SIV H1N1-specific humoral responses in pigs.

Protective efficacy of recombinant H1

To determine the utility of the recombinant H1 vaccines in protecting pigs from infection with a heterologous SIV H1N1, pigs in groups 1-4 were challenged with SIV H1N1 (A/Swine/Indiana/1726/88) fourteen days after the second vaccination. The pigs were monitored for clinical signs, pyrexia, and weight gain for five days post challenge after which they were euthanized and necropsied. No clinical signs were observed prior to challenge at Day 35. During the challenge period, minimal clinical signs were observed with only a few pigs displaying elevated respiration or behavioral signs associated with influenza infection noted across all challenged groups. Average rectal temperatures were stable across the groups with no increases noted during challenge. In addition, weight gain during challenge was not significantly different between the vaccine and control groups. Although the challenge control and H1-Fgag groups gained numerically less weight on average during the challenge period, one-way ANOVA analysis suggested no difference between any of the groups (Fig 5).

In addition to clinical observations, nasal swabs were collected during the challenge period and evaluated by an HA assay to assess the effect of the recombinant H1 vaccines on shedding of the challenge virus. SIV was detected in nasal swab fluid 2 days post challenge (2DPC) in the

placebo group and was detectable in all pigs in this group by 3DPC. All placebo vaccinated pigs remained SIV-positive until necropsy. Detection of SIV in the nasal swabs was delayed and reduced for the H1-2aFc group. Peak detection of SIV in the nasal swabs for this group was 3 out of 10 pigs at 5DPC. The H1-Fgag group showed reduced and sporadic detection of SIV in the nasal swabs while no SIV-positive nasal swabs were detected in the H1-BD and Control groups (Fig 6).

At necropsy, the lungs were examined for lesions consistent with SIV infection and BAL fluids were collected to evaluate for the presence of SIV using a HA assay. Moderate to severe lung lesions consistent with SIV infection were found in the lungs of all pigs in the placebo group at necropsy (Fig 7A). In addition, SIV was detected in BAL fluid from all pigs in this group (Fig 7B). In contrast to the placebo group, only four of ten pigs in the H1-2aFc group and six of ten pigs in the H1-BD and H1-Fgag vaccinated groups were found to have lung lesions. These lesions were mild and typically confined to a single lobe. The mean lung lesion scores for each of these groups was found to be significantly different from the placebo group ($P < 0.0001$) but not from each other (Fig 7A). In addition to reduced lung lesion scores, the number of pigs with detectable SIV in their BAL fluids was also reduced for the H1-2aFc, H1-BD, and H1-Fgag groups with no virus detected in any of the BAL fluids from the pigs in the H1-BD group (Fig 7B).

Discussion

As the goal of a universal influenza vaccine remains elusive, the ability to quickly develop vaccines to respond to emerging influenza strains is of great importance. Recombinant vaccines based on HA alone or in combination with other influenza proteins represent a rapid and

powerful option for the development of vaccines to combat influenza outbreaks. With the previous reports of efficacy in mice utilizing purified subunit HA, VLP displayed HA, and baculovirus displayed HA, the goal of the current study was to evaluate the utility of these approaches for production of efficacious SIV vaccines for swine where protective efficacy was defined as a significant reduction in lung lesions associated with SIV infection. The results of the study demonstrate that vaccines formulated with recombinant H1 produced in the context of a FeLV gag VLP, displayed in the baculovirus envelope, or purified as a subunit IgG fusion protein provide protection of naïve pigs against heterologous H1N1 SIV challenge. Pigs in the recombinant HA vaccinated groups developed SIV H1N1 HI titers and demonstrated significantly reduced lung lesions following challenge. In addition, the number of pigs with SIV detected in the lungs and nasal passages was reduced in these groups when compared to the placebo vaccinated group. To our knowledge, this is the first report detailing the efficacy of these recombinant approaches in pigs.

Secreted HA is a favorable antigen for swine influenza vaccines as the additional processing steps required to extract and purify membrane-associated HA from insect cells, as has been described for human vaccines, can become cost prohibitive for veterinary products [22]. As oligomeric HA has been shown to be more immunogenic than monomer HA, secreted forms of HA require modifications to potentially oligomerize or increase their immunogenicity by other means such as increasing antigen half-life or enhancing uptake by antigen presenting cells [23;24]. Similar to reports of previous work in mouse models, our study demonstrates that HA fused to an IgG Fc domain elicits robust HI antibody titers in pigs and provides protection against challenge with a heterologous H1α cluster SIV [9;25;26]. Interestingly, we found that the HA-2aFc vaccine generated significantly increased HI titers and provided pigs with

protection comparable to that elicited by the baculovirus-displayed and FeLV gag VLP-displayed HA vaccines. These results are surprising given that VLP-based influenza vaccines have been reported to provide superior protection against influenza strains in mice when compared with purified recombinant HA vaccines [27]. As the H1-BD and H1-Fgag vaccines were formulated using hemagglutination titers and the H1-2aFc vaccine was formulated based on gel densitometry measurements, it's difficult to say if this finding is due to the immunogenic properties of the H1-2aFc antigen itself or a difference in the HA content between the vaccines.

Despite comparable protective efficacy in pigs, the baculovirus-displayed HA and FeLV gag VLP-displayed HA vaccines may be a more feasible option for SIV vaccine development. Although an accurate estimation of HA content could not be established using gel densitometry methods for the H1-BD and H1-Fgag antigens, given their unpurified state, SDS-PAGE evaluation of all three antigens suggested that the H1-2aFc vaccine may have contained a greater HA input (data not shown). In addition, the secreted H1-2aFc protein in this study was purified by protein A affinity chromatography as a means to concentrate the antigen due to low yields from the BacDB-H1-2aFc-infected SF+ cells. Despite reported yields of 2 - 5mg/L of a H4-human Fc fusion protein using the BEVS, the process reported in this work would require further optimization for commercial applications as yields were in the hundreds of micrograms per liter range, necessitating large volumes of harvest material to produce sufficient antigen for the trial [9]. In contrast to the H1-2aFc antigen, the baculovirus-displayed and VLP-displayed HA antigens evaluated in this study were utilized directly from harvest supernatant and not purified via sucrose gradient centrifugation as has been previously described for multiple studies [18;28-32]. The alleviation of the need for further processing of these particle-based methods allows for more streamlined and cost-efficient production processes that are vital for veterinary vaccines.

One limitation of this study is that the baculovirus harvests used for the H1-BD and H1-Fgag vaccines were not inactivated prior to formulation. A previous study showed that mice inoculated intranasally with live wild-type baculovirus were protected from lethal challenge with H1N1 influenza virus [33]. Protection from influenza challenge was found to be a result of robust innate immune responses and occurred only when $1.1x10^8$ PFU wild-type baculovirus was administered intranasally 24 hours prior to challenge. When mice were vaccinated subcutaneously or intramuscularly with wild-type baculovirus, no protection was observed. As the pigs treated with the H1-BD and H1-Fgag vaccines were immunized intramuscularly and challenged 14 days after the second vaccination, the probability that the vaccine effects observed in this study were the product of swine innate responses alone, as was described previously for mice, is expected to be low. In addition, SIV H1-specific humoral responses were detected in the serum of pigs immunized with the H1-BD and H1-Fgag vaccines further confirming the specificity of the immune response generated by these vaccines.

Utilization of the BEVS has a number of advantages in the development of SIV vaccines. With the commercial availability of a variety of cloning vectors and baculovirus DNA preparations that minimize non-recombinant baculovirus generation during co-transfection, the time needed to generate recombinant baculovirus clones has been greatly minimized. In addition, insect cells can be propagated in suspension culture without serum allowing for straightforward scale-up and cost effective antigen production at manufacturing scale [34]. The safety profile for products derived from the system is also well established as licensed vaccines for porcine circovirus and classical swine fever virus are currently produced using the BEVS [35]. Recently, the USDA has adopted revised licensing requirements allowing for rapid licensing of new products using existing expression platforms like the BEVS [36]. These new regulations coupled

with the additional advantages presented above make the BEVS an attractive platform for the development of SIV vaccines for swine.

In conclusion, the results of this study build on previously published reports demonstrating that HA expressed with the BEVS generates protective responses in birds, mice, and other mammals. With a single report of efficacy in pigs utilizing only an enveloped VLP approach, the current findings expand the utility of the BEVS by demonstrating that recombinant HA expressed as a soluble subunit, displayed in an enveloped VLP or displayed in the baculovirus envelope provide protection of swine against SIV H1N1 challenge. The results suggest that these recombinant approaches utilizing the BEVS may be a viable option for the development of SIV vaccines for swine.

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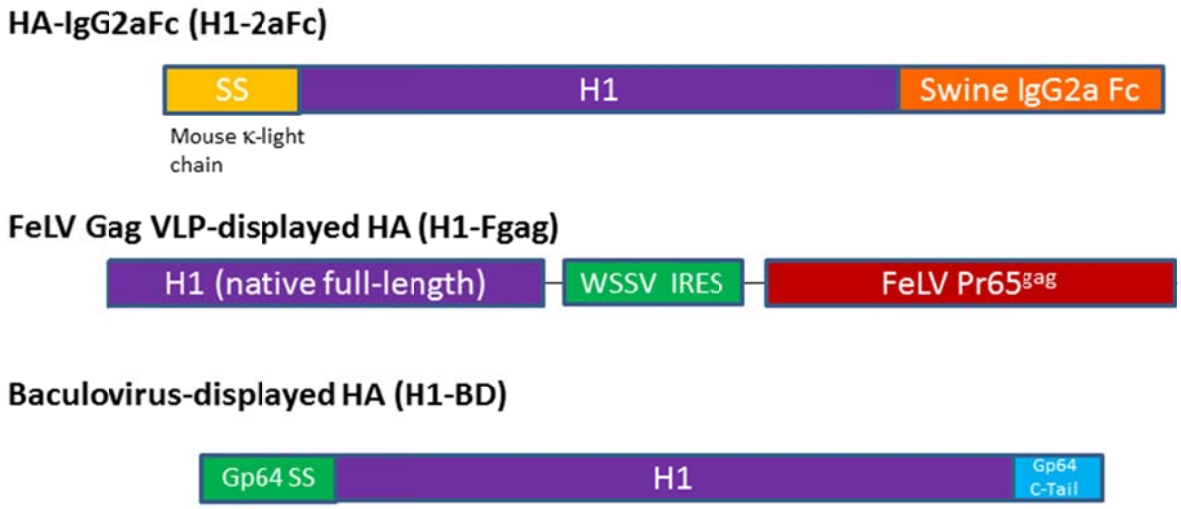

Fig 1. Schematic of coding sequences for HA constructs.

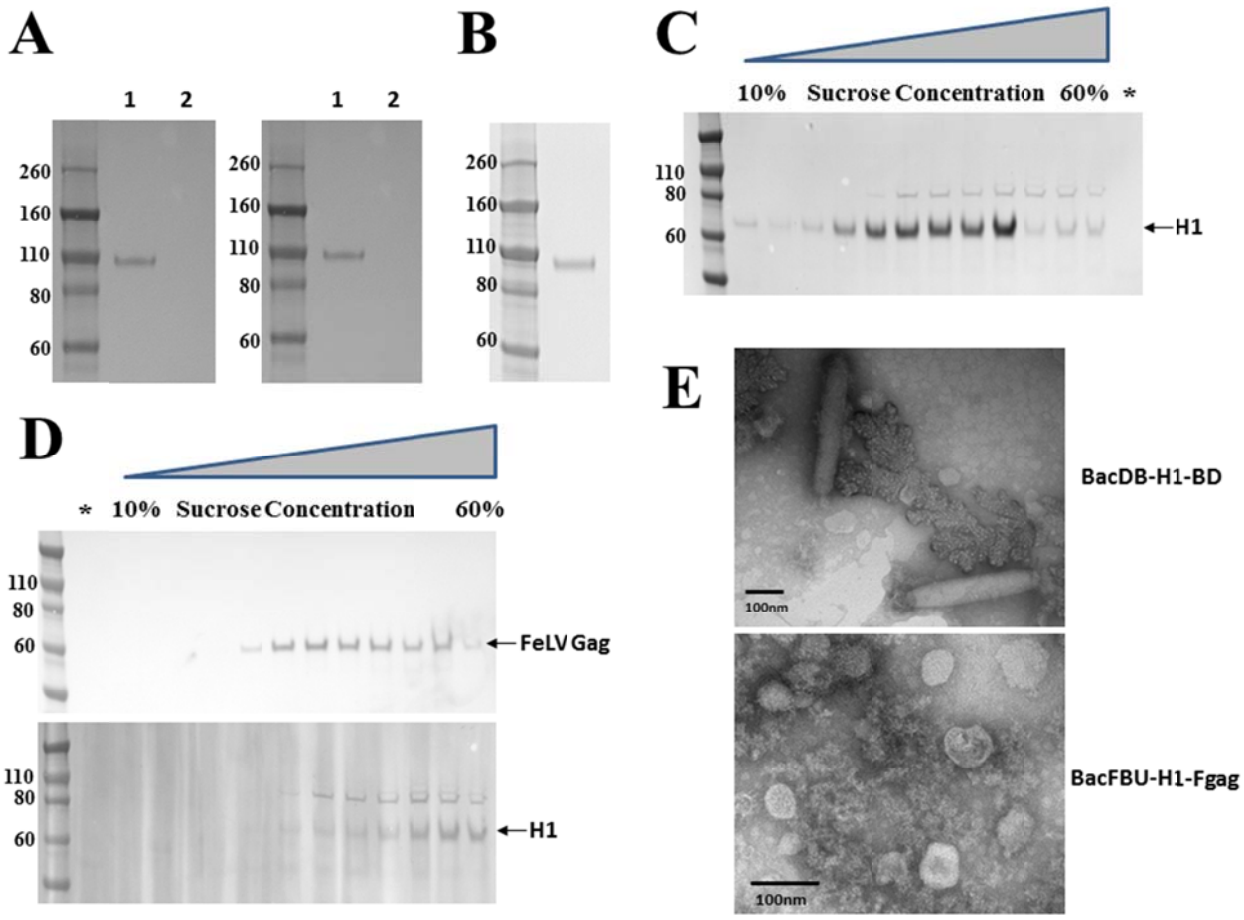

Fig. 2. Evaluation of H1 expressed from recombinant baculovirus. (A) Western blots of BacDB-H1-2aFc harvest supernatant (1) or control baculovirus supernatant (2) with H1-specific (left panel) or swine IgG-specific (right panel) antibodies. (B) Protein A purified H1-2aFc separated by SDS-PAGE. Expected molecular weight of H1-2aFc from amino acid sequence is 88kDa. Resuspended ultracentrifugation pellets from BacDB-H1-BD and BacFBU-H1-Fgag harvest supernatants were separated on sucrose gradients. (C) Western blot of BacDB-H1-BD gradient fractions with H1-specific antibodies. (D) Western blot of BacFBU-H1-Fgag gradient fractions with FeLV Gag-specific (upper panel) or H1-specific (lower panel) antibodies. Lanes with * contain resuspended ultracentrifugation pellets from control baculovirus. (E) Electron micrographs of negative stained pooled sucrose fractions from BacDB-H1-BD or BacFBU-H1-Fgag harvests.

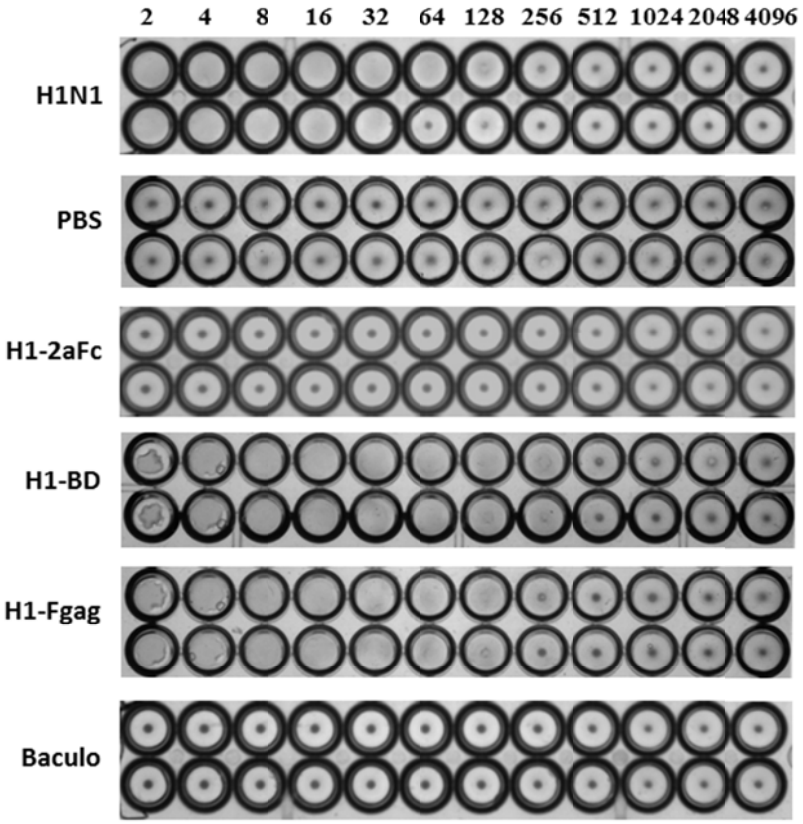

Fig. 3. Hemagglutination activity of H1 antigens. Recombinant H1 antigens were tested for HA activity using turkey red blood cells in an HA assay. SIV H1N1 A/Swine/Indiana/1726/88 (H1N1) and PBS were tested as positive and negative controls respectively. H1-2aFc was tested after protein A purification while H1-BD, H1-Fgag, and a control baculovirus (Baculo) were tested as harvest supernatants.

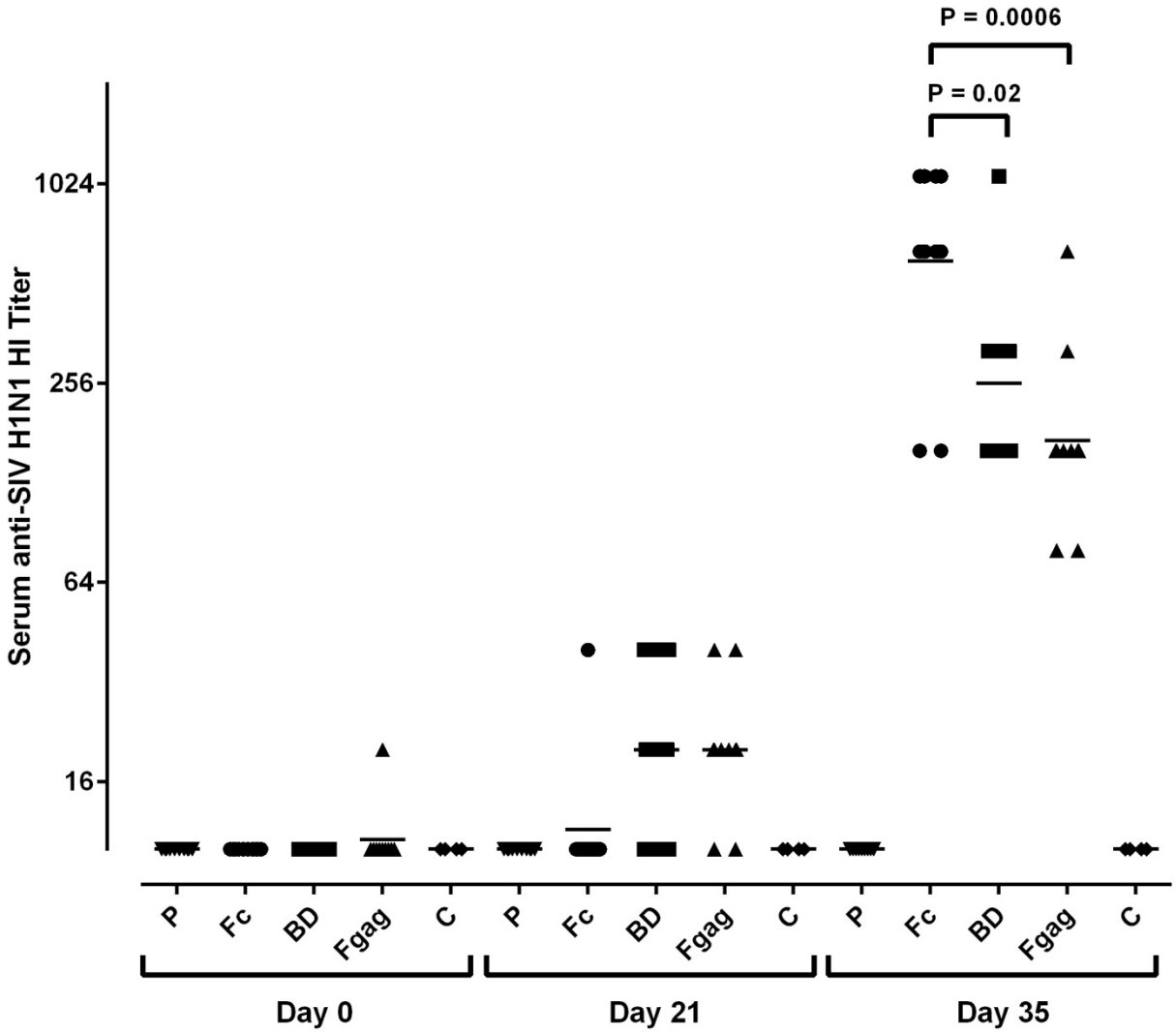

Fig. 4. Development of HI antibody titers after vaccination. Pigs were vaccinated on day 0 and 21 with H1-2aFc (Fc), H1-BD (BD), or H1-Fgag (Fgag) experimental vaccines or placebo (P). Control (C) pigs were not vaccinated. Serum samples were evaluated for HI activity against SIV H1N1 A/Swine/Indiana/1726/88 using an HI assay. Symbols represent individual pigs while bars represent group geometric means.

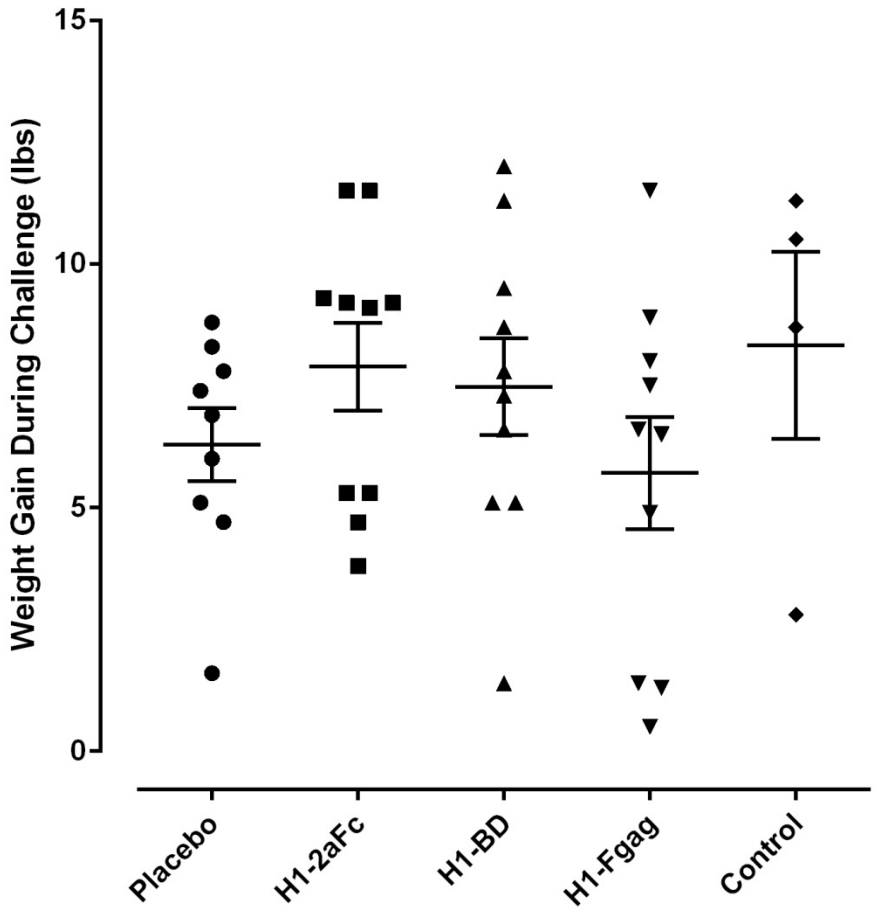

Fig. 5. Weight gain of pigs during challenge period. Pigs were weighed on the day of challenge and the day of necropsy. The points represent the difference in weight between the two days for each pig with mean and standard error depicted by the bars.

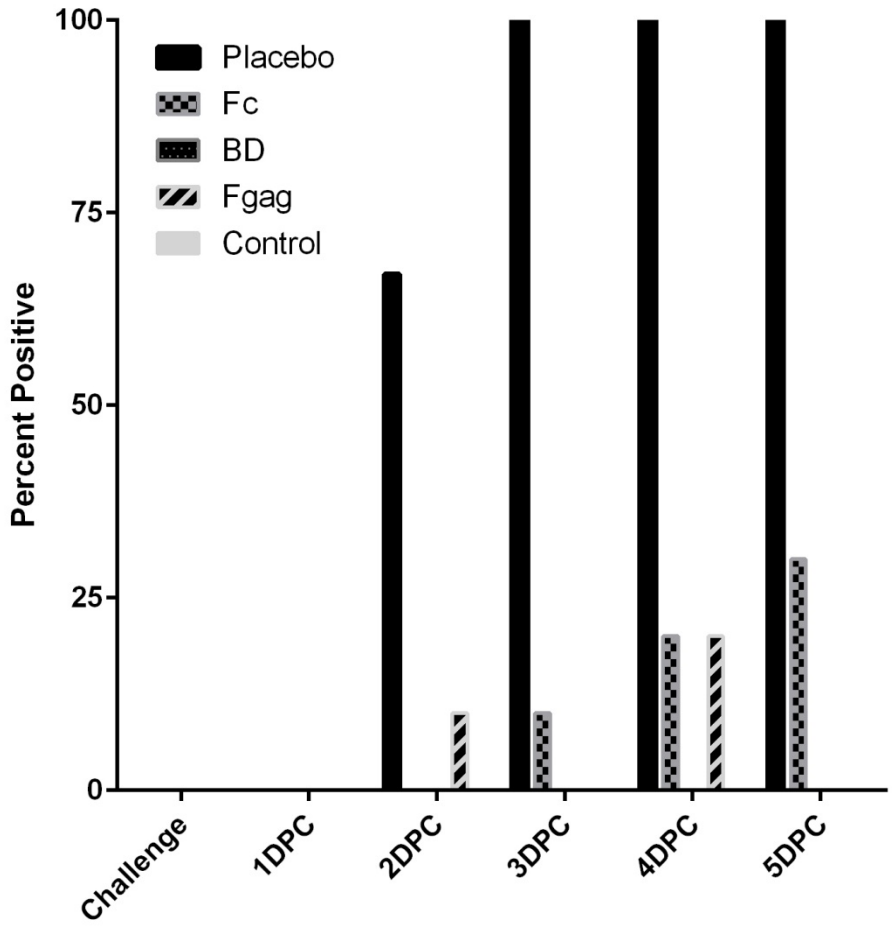

Fig. 6. Viral shedding during challenge. Nasal swabs were collected from each pig daily during the challenge period. The presence of SIV in swab fluids was detected using an HA assay after one passage on MDCK cells.

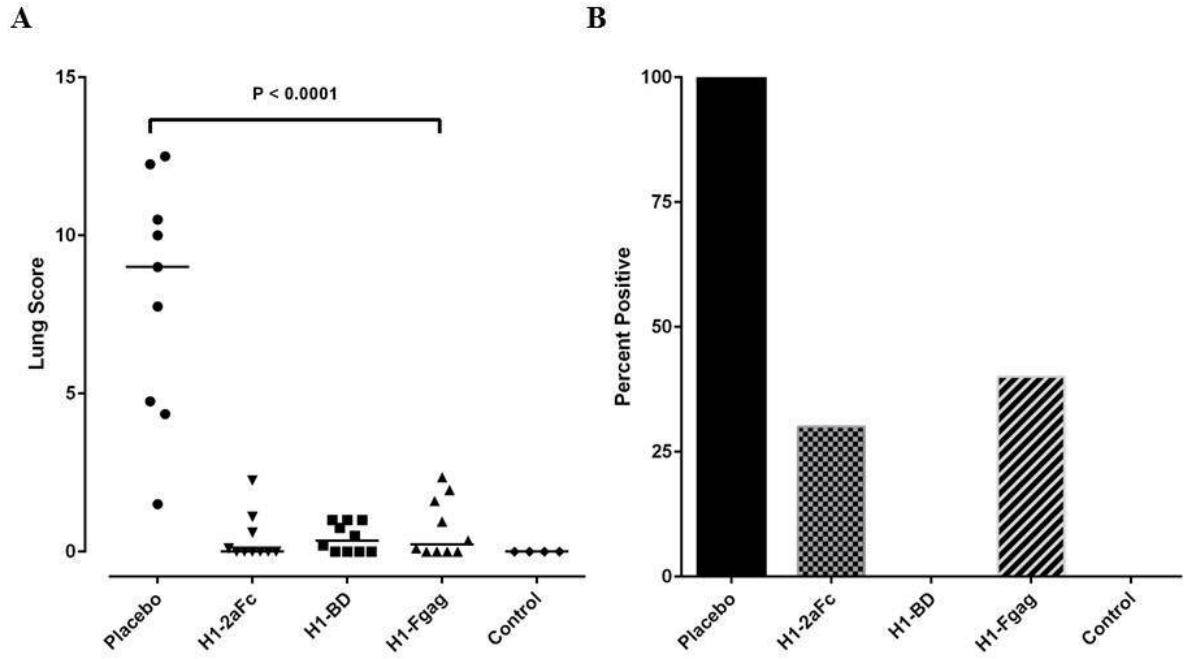

Fig 7. Lung scores and presence of SIV in the lungs after challenge. At necropsy, the lungs of pigs from each group were evaluated for lesions typical of SIV infection and scored as described in the Materials and Methods. (A) Lung scores are presented as individual points with bars indicating group medians. After lung scoring, BAL fluids were collected and passaged once in chicken eggs. (B) SIV was detected in the resulting allantoic fluid using an HA assay.

Table 1. Efficacy study design.

Group	# of Pigs	Treatments (D0 & D21)	Challenge (D35)	Necropsy (D40)
1 (Placebo)	10	Sf900III/TBS + Emulsigen D		
2	10	H1-2aFc[a] + Emulsigen D	2mL dose IT of SIV H1N1 A/Swine/Indiana/ 1726/88[c]	Lungs collected for BAL and gross lung lesion evaluation
3	10	H1-BD[b] + Emulsigen D		
4	10	H1-Fgag[b] + Emulsigen D		
5 (Control)	4	N/A	N/A	

[a] 200µg per dose
[b] Formulated with 2048 HAU per dose
[c] 7.78×10^5 EID_{50}/mL

CHAPTER 3: RESCUE OF BACULOVIRUS-EXPRESSED PORCINE CIRCOVIRUS TYPE 2B VIRUS-LIKE PARTICLE PRODUCTION WITH SINGLE AMINO ACID MUTATION

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Abstract

Porcine Circovirus type 2 (PCV2) remains an economically important disease agent in the swine industry despite the availability of efficacious vaccines. A number of variant strains, classified as PCV2b, have recently been described globally, potentially due to the increasing pressure from vaccination programs. The emergence of these novel strains of PCV2 underline the importance of continued vaccine development incorporating efficacious antigens from circulating and emerging strains of the virus. The present study details the development of an experimental PCV2 vaccine based on the capsid protein (ORF2) from an emerging strain, PCV2b BDH, expressed as a virus-like particle (VLP) in insect cells using the Baculovirus expression vector system (BEVS). The results of the study demonstrate that yield of PCV2b BDH ORF2 (ORF2b) VLP antigen in baculovirus-infected insect cells is greatly reduced when compared to PCV2a ORF2 (ORF2a). Mutational analysis of the ORF2b protein revealed that ORF2b VLP antigen production could be restored to levels similar to those produced from an ORF2a-expressing

baculovirus construct when the arginine at position 63 was replaced with a non-basic amino acid such as threonine, aspartic acid, glutamine, leucine or glycine. Additionally, an experimental vaccine utilizing one of the modified capsid proteins, ORF2b R63T, prepared in the BEVS was successful in reducing viremia, colonization of lymphoid tissue, and lymphoid depletion and inflammation upon challenge with virulent PCV2b. Taken together, the present study identifies a potential problem for the future development of VLP-based PCV2 vaccines using the BEVS and details methods to overcome this problem.

Introduction

Porcine Circovirus disease (PCVD) continues to be a serious problem among the swine producing regions of the world. Clinical manifestations of PCVD can include wasting, respiratory disease, dermatitis, enteritis, and occasionally reproductive disease [1-3]. Because of the economic impact of PCVD on the global swine industry, a number of efficacious porcine circovirus type 2 (PCV2) vaccines, consisting of inactivated PCV2 virus or PCV2 ORF2 virus-like particles (VLPs) generated in insect cells utilizing the baculovirus expression vector system (BEVS), became available in the mid-2000s [4]. The formulation of these vaccines utilized antigen from the historically predominant PCV2a genotype. Just prior to the commercial release of these vaccines, a shift in the genotype of the circulating strains from PCV2a to PCV2b was reported by multiple sources with PCV2b isolates more highly associated with the wasting disease caused by PCV2 systemic infection (PCV2-SD) [5;6]. Despite this shift in the predominant PCV2 genotype, the currently available vaccines have been shown to be effective against the most recent circulating PCV2b strains and remain in intense use today [7-9].

Over the last five years, novel PCV2b isolates associated with PCVD manifestation on vaccinated farms have been reported globally [10-14]. In addition, recombination between

PCV2 strains both *in vitro* and in the field has been documented [15-17]. Furthermore, phylogenetic analyses of PCV2 genomic sequences have demonstrated a nucleotide substitution rate higher than most DNA viruses and near that of single-stranded RNA viruses, highlighting the plasticity of the PCV2 genome [18;19]. As the currently available PCV2a-based vaccines do not induce sterilizing immunity, the heavy evolutionary pressure from global vaccination strategies coupled with the plasticity of the PCV2 genome may result in the accelerated generation of novel vaccine-escape strains, emphasizing the need for development of new vaccines containing antigen from emerging PCV2 strains.

PCV2 strain BDH is a representative isolate of an emerging cluster within the PCV2b genotype associated with recent vaccine breaks in Asia, South America, North America and Europe [10;20]. When PCV2b BDH ORF2 (ORF2b) was expressed using the BEVS, an important commercial production platform for ORF2 VLP-based PCV2 vaccines, yields of ORF2b VLPs were found to be greatly decreased when compared to the yield of PCV2a ORF2 (ORF2a) VLPs. This finding was surprising given the high nucleotide and amino acid identity between the two ORF2 proteins. The current report details an investigation into the mechanisms associated with the diminished yield of ORF2b VLPs and further details the steps implemented to rescue ORF2b VLP yield while retaining the immunogenic and efficacious properties of the VLP. Although multiple reports suggest that the currently available PCV2a-based vaccines are efficacious against the more recent BDH-like strains, the resolution of the hurdles described herein will have implications for the future development of VLP-based vaccines against strains of PCV2 that may be resistant to the currently available vaccines [21;22].

Materials and Methods

Cells and viruses

Semi-adherent *Spodoptera frugiperda* (Sf9) cells were used for generation and amplification of recombinant baculoviruses. Sf9 cells were maintained in BaculoGold TNM-FH medium (Becton Dickinson, Franklin Lakes, NJ) at 28^{o}C. Expression evaluation of recombinant baculovirus was completed using serum-free suspension-adapted *Spodoptera frugiperda* cells (SF+) maintained in Sf900III 1X SFM (Life Technologies, Norwalk, CT) at 28^{o}C in spinner flasks with agitation at 100rpm. PCV2b challenge virus was isolated from the lung of an infected pig and subsequently propagated on VIDO-R1 cells maintained in MEM at 37^{o}C with 5% CO2.

Generation of recombinant baculoviruses

The coding sequences for PCV2b BDH ORF2 (Genbank accession# HM038017) and subsequent mutants were synthesized at Integrated DNA Technologies (IDT, Iowa City, IA) and cloned into baculovirus transfer vector pVL1393 via BamHI and NotI restriction sites. The PCV2a ORF2 T63R mutant coding sequence was prepared by site-directed mutagenesis of plasmid pVL1392-ORF2a, yielding pVL1392-ORF2a T63R, using the QuikChange Lightning Site-Directed Mutagenesis kit (Agilent Technologies, Santa Clara, CA).

Recombinant baculovirus were prepared via co-transfection of Sf9 cells with linearized FlashBAC ULTRA (Oxford Expression Technologies) or BaculoGold (Becton Dickinson) baculovirus DNA and the respective baculovirus transfer plasmids containing the appropriate PCV2 ORF2 coding sequence under the control of the polyhedrin promoter. Co-transfection supernatants were harvested after 5 days incubation at 28^{o}C and clarified by centrifugation at

1000 x g to separate the remaining cells from the viral transfection harvest fluids. Collected cells from the co-transfections were evaluated for PCV2 ORF2 expression by an immunofluorescence assay (IFA) with PCV2-specific antibodies. Viral transfection harvest fluids were 0.2μm filtered and subsequently amplified on Sf9 cells to generate P2 viral stocks. Amplified stocks of recombinant baculovirus were titered via a fluorescent antibody infectious dose 50 ($FAID_{50}$) assay utilizing baculovirus gp64-specific monoclonal antibody (eBioscience, San Diego, CA).

Preparation of culture samples and harvest fluids

To prepare culture samples, baculovirus-infected SF+ cells were harvested at various time points and clarified by centrifugation at 1000 x g for five minutes. The resulting cell pellets and supernatants were frozen separately at -70^{o}C. For the preparation of total cell lysates, frozen cell pellets were thawed and resuspended in lysis buffer (20mM Tris-HCl, 1% Triton X-100, 250u/mL benzonase, 10μL/mL protease inhibitor cocktail) at a concentration of $4x10^{6}$ cells/mL, vortexed for 10 seconds, subsequently incubated for five minutes at room temperature on a rocking platform, and then vortexed a second time. Total cell lysates were separated into soluble (supernatant) and insoluble (pellet) fractions by centrifugation at 19,000 x g for 10 minutes at 4^{o}C. Total cell lysates and soluble fractions were supplemented with reducing sample buffer and heated to 98^{o}C for 10 minutes. The insoluble pellets were extracted directly in reducing sample buffer for 10 minutes at 98^{o}C. The denatured samples were immediately evaluated by Western blot or stored at -70^{o}C.

Harvest fluids were prepared from infected SF+ cultures at 5 to 7 days post-infection, when cell viability dropped below 30% as measured using a Vi-CELL Cell Viability Analyzer (Beckman Coulter, Jersey City, NJ). Infected SF+ cultures were centrifuged at 20,000 x g for 20

minutes at 4°C to pellet cells and insoluble debris. Supernatants were then 0.2μm filtered and stored at 4°C.

SDS-PAGE and Western blot

Samples were prepared in 1X sample buffer and separated by SDS-PAGE under reducing conditions on 4-12% polyacrylamide pre-cast gels (NuPAGE system, Life Technologies, Norwalk, CT). Gels were stained directly using SimplyBlue™ Safe Stain (Life Technologies) or transferred to nitrocellulose membranes. For Western blots, membranes were blocked and then probed with either PCV2-specific purified swine IgG or mouse monoclonal antibody to actin (Thermo Scientific, Waltham, MA) followed by incubation with peroxidase-labeled anti-swine IgG or anti-mouse IgG secondary antibodies (Jackson ImmunoResearch, West Grove, PA). Membranes were developed with TMB membrane substrate (KPL, Gaithersburg, MD) and imaged using a FluorChem™ HD camera (Protein Simple, San Jose, CA).

Quantitation of PCV2 ORF2 virus-like particles

Baculovirus harvest fluids were subjected to centrifugation at 100,000 x g for 2 hours at 4°C to pellet PCV2 ORF2 virus-like particles (VLPs). The resulting pellets were resuspended in TBS and further separated on a 10% - 60% discontinuous sucrose gradient at 100,000 x g for 2hrs at 4°C. The fractions containing the majority of the PCV2 ORF2, as determined by SDS-PAGE and Western blot, were pooled and evaluated by densitometry. Briefly, pooled PCV2 ORF2-containing fractions were separated by SDS-PAGE as described in the previous section with gel images evaluated using AlphaView software (Protein Simple). The mass of PCV2 ORF2 bands were calculated using a BSA standard curve included on each gel. The PCV2 ORF2 concentration of the pool was calculated by dividing the mass of the PCV2 ORF2 band(s) by the

total volume of sample loaded on the gel. PCV2 ORF2 concentrations in harvest material were calculated by multiplying the PCV2 ORF2 concentration in the pool by the volume of the pool and then dividing the result by the starting volume of harvest fluids used for centrifugation.

Confirmation of VLP formation by electron microscopy

Pooled PCV2 ORF2-containing fractions were dialyzed against TBS, pH7.5 to remove the sucrose and then concentrated using VivaSpin 100,000 MWCO spin concentrators (Sartorius Stedim). Concentrated samples were submitted for negative stain transmission electron microscopy (TEM) evaluation at the National Animal Disease Center (NADC), Ames, IA.

Animals and study design

Fifty-four cesarean-derived colostrum-deprived (CDCD) healthy commercial mix pigs, three weeks of age, were randomized into three treatment groups blocked by litter (Table 1). Pigs were housed in six pens in a single room with equal distribution of treatment groups among pens. On day 0, all pigs in Groups 2 and 3 were vaccinated with an ORF2b R63T vaccine or an ORF2a vaccine respectively. Subsequently, pigs in Groups 1 -3 were challenged on day 14 with 5.25 $\log_{10}$ tissue culture infectious dose 50 ($TCID_{50}$) PCV2b in a 2mL dose (1mL intranasal and 1mL intramuscular). One pig in Group 1 and two pigs in Group 2 died prior to challenge. On days 11 and 17 keyhole-limpet hemocyanin (KLH), 1 mg/mL, emulsified in incomplete Freund's adjuvant was administered in alternating ham muscles of all pigs in groups 1 - 3. During the 28 day challenge period pigs were monitored for clinical signs daily. Pig weights were collected on days 0, 14, and 42 while serum samples were taken on days 0, 14, 21, 28, 35, and 42.

At day 42 all animals were humanely euthanized by electrocution while under anesthesia (1mg/lb xylazine and 10mg/lb ketamine) and samples of tonsil, tracheobronchial lymph node

(TBLN), mesenteric lymph node (MLN), and iliac lymph node (ILN) were collected and stored in 10% buffered formalin. Formalin-fixed tissue samples were submitted to the Iowa State University Veterinary Diagnostic Lab (ISU-VDL) for microscopic evaluation by a pathologist and tissue samples were scored from 0 (normal) to 3 (severe) for lymphoid inflammation, lymphoid depletion and the presence of PCV2 antigen by immunohistochemistry (IHC). The study protocol was approved by the Boehringer Ingelheim Vetmedica, Inc. Institutional Animal Care and Use Committee.

Formulation and administration of vaccines

ORF2b R63T and ORF2a VLPs were generated in SF+ cells through infection with recombinant baculovirus expressing the respective ORF2 proteins under the control of the polyhedrin promoter. The vaccines were formulated with inactivated harvest fluids to contain 13μg of respective VLP per dose in adjuvant. Vaccines were administered intramuscularly in the left side of the neck on Day 0. No placebo vaccine was given to the challenge control pigs in Group 1 as multiple studies to support licensing of Ingelvac CircoFLEX® have demonstrated no effect of placebo vaccination.

Serology

Sera samples were assessed for the presence of anti-ORF2a or anti-ORF2b IgG antibodies prior to and after vaccination using an indirect ELISA assay. Briefly, wells of 96 well plates were coated overnight at 4^{o}C with 50ng of purified VLPs (ORF2a or ORF2b R63T) in 0.1M carbonate buffer, pH 9.6 (100μL/well). After removal of the coating antigen, wells were blocked with 150μL/well casein blocker (Thermo Scientific) for 1hr at room temperature. Serum samples and a positive control sample (PCV2-specific purified swine IgG) were tested in

duplicate wells (100μL/well) at a 1:200 dilution in casein blocker (100μL/well) at 37^{o}C with agitation at 100rpm. Bound IgG was detected with peroxidase-labeled goat anti-swine IgG secondary antibody (Jackson ImmunoResearch, West Grove, PA) at 100μL/well using the same incubation conditions as the test samples followed by development with TMB substrate (KPL, Gaithersburg, MD) at room temperature(100μL/well). Plates were washed 5X with 300μL/well TBST (20mM Tris-HCl, 500mM NaCl, 0.05% Tween 20, pH 7.5) on a plate washer (BioTek, Winooski, VT) after the coating antigen, primary, and secondary antibody steps. Sera samples from the untreated control group were tested on plates coated with ORF2a VLPs and plates coated with ORF2b R63T VLPs. Sera samples from the ORF2a-vaccinated group and the ORF2b R63T-vaccinated group were tested on plates coated with their respective immunizing antigens.

Quantitative PCR

For detection of PCV2 DNA in serum samples, total nucleic acid was extracted on a BioSprint 96 workstation using the One-for-All Vet kit (Qiagen, Valencia, CA). PCV2b genomic DNA in extracts was detected by a quantitative real-time PCR (qPCR) assay using specific primers and probe (Table 2) [23]. Real-time PCR was carried out in 20μL reactions containing 10μL 2X SsoAdvanced universal probes supermix (Bio-Rad, Hercules, CA), 0.25μM of each primer, 0.2μM probe and 2μL of extracted nucleic acid sample. Reactions were incubated at 95^{o}C for 2 minutes then cycled 40X between 95^{o}C for 5 seconds and 60^{o}C for 5 seconds in a CFX96 thermocycler (Bio-Rad). Threshold values were converted to genomic copies using a standard curve derived from single-stranded oligonucleotides containing the target amplicon.

For assessment of PCV2a ORF2 and PCV2b ORF2 messenger RNA (mRNA) levels from baculovirus-infected insect cells, cell pellets were processed for total RNA using a QIAshredder and RNeasy Plus mini kit (Qiagen). Specific mRNA levels in total RNA extracts were determined by quantitative reverse transcriptase PCR (qRT-PCR) using primer and probes sets for detection of *Spodoptera frugiperda* 28s ribosomal RNA (rRNA), baculovirus gp64, PCV2a ORF2 and PCV2b ORF2 (Table 2)[24;25]. Reactions were carried using the iTaq Universal Probes One-Step kit (Bio-Rad) following the manufacturer's directions in a 20μL reaction containing an appropriate concentration of primers and probe (Table 2) and 100pg of RNA sample. Reactions were incubated at 50^{o}C for 15 minutes, heated to 95^{o}C for 5 minutes, then cycled 40X between 95^{o}C for 15 seconds and 60^{o}C for 15 seconds in a CFX96 thermocycler (Bio-Rad). All RNA samples were tested in triplicate. Relative transcript levels for each gene were determined using the $2^{-\Delta\Delta CT}$ method normalizing all C_T values to 28s rRNA and reporting PCV2a ORF2 and PCV2b ORF2 transcript levels as fold-increase over baculovirus gp64 transcript levels [26]. Prior to employing $2^{-\Delta\Delta CT}$ quantitation methods, amplification efficiency for each primer/probe set was calculated using a 2-fold series of RNA input levels from 1.6ng to 12.5pg and plotting C_T values vs. $\log_{10}$pg of input RNA. Amplification efficiency for all primer/probe sets was between 97% and 100%.

Statistics

Analysis of the incidence of lymphoid depletion, lymphoid inflammation, and IHC scores was completed designating an animal as affected for an endpoint if one or more tissues were scored as abnormal (score of 1 or higher). The outcome (normal/abnormal) was then analyzed using generalized linear mixed model (binomial distribution with a logit link function) with a fixed effect for treatment and a random effect representing the pen an animal was housed in during the

challenge phase of the study. P-values associated with testing for group differences were adjusted for multiplicity using the simulated distribution of the of a multivariate *t* random vector [27]. To assess differences in the severity of lymphoid lesions between the vaccinated and untreated control groups, mitigated fractions with 95% confidence intervals for maximum scores for lymphoid depletion, lymphoid inflammation, and quantity of PCV2 antigen detected by IHC in the tonsil and lymph nodes (TBLN, ILN, and MLN) were used. Mitigated fractions describe the probability that an intervention will decrease the severity of a disease outcome when compared to the disease outcome with no intervention [28]. The 95% confidence intervals for the mitigated fractions were estimated using bootstrapping methods, stratifying by pen. All analyses were conducted with SAS® Version 9.4.

Results

Confirmation of VLP formation by baculovirus expressed ORF2b

Infection of insect cells with baculovirus encoding ORF2a results in expression and assembly of ORF2a into VLPs. As the baculovirus infection progresses, increasing numbers of insect cells are lysed, releasing the ORF2a VLPs into the culture supernatant. To determine if infection of insect cells with recombinant baculovirus expressing ORF2b resulted in the generation of ORF2b VLPs in the culture supernatant, similar to ORF2a, SF+ cells were infected at a multiplicity of infection (MOI) of 0.1 and culture supernatants were evaluated by Western blot and processed for TEM. The Western blot results showed a band of the expected size in culture supernatants from Day 3 post-infection onward (Fig 1A). Surprisingly, the relative abundance of ORF2b in the culture supernatant was considerably reduced when compared to the abundance of ORF2a in culture supernatant from SF+ cells infected with baculovirus encoding ORF2a. Evaluation of the

ORF2b present in culture supernatant at Day 5 post-infection by sucrose gradient centrifugation and TEM revealed VLPs of approximately 20nm in size, similar to those formed by ORF2a (Fig 1B & C). Quantitation of the ORF2b VLPs demonstrated that ORF2b VLP yield in the culture supernatant was 14-fold lower than ORF2a VLP yield from ORF2a-encoding baculovirus. These findings were unexpected given that the ORF2a and ORF2b proteins share 91% amino acid identity and their coding regions share 91% nucleic acid identity.

Evaluation of expression of ORF2b in recombinant baculovirus-infected insect cells

To determine if the low ORF2b VLP yield in the supernatant was a result of lower levels of ORF2b expression, SF+ cells were infected with recombinant baculovirus encoding ORF2b or ORF2a at a MOI of 0.1 and cells were harvested daily during the infection. Analysis of ORF2a and ORF2b transcription levels by qRT-PCR demonstrated a 10 – 15-fold relative increase over gp64 for ORF2a from day 3 to day 5 post-infection while ORF2b showed at most a 1.8-fold increase over gp64 during the same time period (Fig 2A). Despite the large increase in ORF2a transcription, analysis of total cell lysates by Western blot demonstrated that protein concentrations were similar between ORF2a and ORF2b during the same time period (Fig 2B). Interestingly, when the total cell lysates were clarified by centrifugation to remove insoluble proteins, the majority of the ORF2b was removed and subsequently detected in the insoluble fraction (Fig 2B). In contrast, a larger amount of the ORF2a remained in the soluble fraction after lysate clarification. These results demonstrate that despite lower relative transcription levels, ORF2b is expressed at levels similar to ORF2a and suggest that the amino acid differences in ORF2b may contribute to the decreased yield of ORF2b VLPs through low solubility in insect cells.

Single amino acid mutation increases ORF2b VLP yield

With the goal of determining whether the amino acid differences in ORF2b were responsible for the decreased ORF2b VLP yield observed with the BEVS, the regions of amino acid heterogeneity between ORF2a and ORF2b were assessed by amino acid sequence alignment (Fig 3). The results of the alignment revealed that seven positions in ORF2b contained amino acids with dissimilar properties when compared to ORF2a. Three of these differences were located on a set of β-hairpin loops predicted to be surface exposed while two of the differences involved proline which is known to induce bend or turns in an amino acid chain (Fig 4). To determine if these amino acid changes contributed to the reduced ORF2b VLP yields in insect cells, recombinant baculovirus encoding mutant ORF2b proteins containing ORF2a substitutions were used to infect SF+ cells at a MOI of 0.1. The resulting harvest supernatants were assessed for VLP formation by TEM and VLP concentration by gel densitometry analysis of pooled sucrose gradient fractions. The yields of VLP for the K59A and N232E mutant ORF2b proteins were similar to those for the native ORF2b protein (Fig 5A). Formation of VLPs for P88K, T151P, G191R and I206K ORF2b mutants could not be determined as culture supernatants from these constructs contained near undetectable levels of ORF2b. In contrast, yields of VLP for the R63T mutant were increased 8-fold over those for the native ORF2b protein. Reciprocally, when culture supernatants from SF+ cells infected with baculovirus encoding a T63R ORF2a mutant were evaluated for VLP yield, a dramatic loss of VLPs was observed (Fig 5B). Taken together, these data demonstrate that ORF2 VLP yield from the BEVS is greatly reduced by the presence of arginine at position 63 of ORF2.

Reduced ORF2b VLP yields with charged or aromatic amino acids at position 63

As position 63 of ORF2 resides in a surface exposed loop, it may be susceptible to genetic variation as PCV2 continues to evolve under heavy vaccine pressure (Fig 6). To determine if the presence of other amino acids at position 63 may cause problems with ORF2 VLP yield similar to that of arginine when produced using the BEVS , recombinant baculoviruses encoding ORF2b mutants interchanging a diverse set of amino acids at position 63 were used to infect SF+ cells at an MOI of 0.1. An evaluation of the resulting harvest supernatants demonstrated that lower VLP yields were observed when lysine, tryptophan, tyrosine, and aspartic acid were present at position 63 (Fig 5B). The results of these experiments suggest that challenges, similar to those described here for ORF2b, may arise during the development of ORF2 VLP-based vaccines for future PCV2 strains when their ORF2 proteins contain basic, acidic or aromatic amino acids at position 63.

Effect of R63T mutation on ORF2b expression

To investigate the mechanism behind the increased VLP yield of the ORF2b R63T mutant, recombinant baculoviruses encoding ORF2a, ORF2b or ORF2b R63T were used to infect SF+ cells at an MOI of 0.1 and cells were harvested daily. When cells pellets were analyzed for transcription levels using a qRT-PCR assay, the levels of ORF2b and ORF2b R63T transcripts relative to gp64 were found to be similar on days 3-5 post infection (1.2 to 2.7-fold increase) while ORF2a transcript levels were again found to be 10 to 15-fold increased over gp64 (Fig 7A). Analysis of total cell lysates by Western blot revealed that the intracellular levels of ORF2a, ORF2b, and ORF2b R63T were similar. When total lysates were clarified by centrifugation, the ORF2b low solubility phenotype was unchanged for ORF2b R63T as the majority of the protein

was removed from the soluble fraction (Fig 7B). These data demonstrate that the mechanism of enhanced VLP yield associated with the ORF2b R63T mutant remains to be elucidated as replacement of arginine with threonine at position 63 does not increase transcription levels and does not abrogate the low solubility phenotype observed for ORF2b.

Modified ORF2b protects against virulent PCV2b challenge

To evaluate the immunogenicity and protective efficacy of the mutant ORF2b protein, pigs were vaccinated with adjuvanted ORF2b R63T antigen or adjuvanted ORF2a antigen and challenged fourteen days later with a virulent PCV2b strain (Table 1). ELISA evaluation of sera samples taken prior to challenge (day 14), revealed that all pigs in both vaccinated groups had developed a humoral response against their respective antigens while no humoral response was detected in the untreated controls (Fig 8). After challenge, clinical signs were observed sporadically in a small fraction of the pigs in all groups consisting of increased respiratory rate, lethargy, depression, lameness, and mild cough. Two of the pigs in the untreated control group displayed the most consistent clinical signs beginning on day 35 and were euthanized on day 39 for humane reasons. A third pig in the untreated control group was found dead on day 40. Assessment of viremia by qPCR revealed that PCV2b DNA was detected in the serum of all pigs on day 21 with fewer pigs positive in the ORF2a and ORF2b R63T vaccinated groups on subsequent days. The ORF2a and ORF2b R63T vaccinated groups demonstrated a 1.7 to 2.9 $\log_{10}$ reduction in the mean PCV2b DNA/mL from day 28 onward when compared to the untreated controls (Fig 9A). In addition, average weight gain during the study was numerically higher for the ORF2a and ORF2b R63T vaccinated groups (Fig 9B).

When lymphoid tissues were examined for the presence of microscopic lesions associated with PCV2 infection, histiocytic to granulomatous inflammation and lymphoid depletion were observed in 82% and 65% of the untreated control pigs respectively with PCV2 antigen detected by IHC in 94% of these pigs (Fig. 10). The incidence of lymphoid inflammation and lymphoid depletion was lower in the vaccinated groups with only 50% displaying lymphoid inflammation and 13% or less displaying lymphoid depletion (6% for ORF2a and 13% for ORF2b R63T). In addition, the detection of PCV2 antigen was reduced to 61% and 56% respectively in the ORF2a and ORF2b R63T groups. The reduction in the incidence of lymphoid depletion in both vaccinated groups was found to be statistically significant (ORF2a, $P = 0.028$, ORF2b R63T, $P = 0.042$). Mitigated fraction analysis of the maximum severity scores demonstrated that the ORF2a and ORF2b R63T vaccines significantly reduced the severity of lymphoid depletion and lymphoid inflammation as well as the quantity of PCV2 antigen detected by IHC. Observed mitigated fractions with 95% confidence intervals are displayed in table 3.

Discussion

Here we describe for the first time the expression and VLP formation of the ORF2 protein from emerging PCV2b strain BDH using the BEVS. The work detailed in this report demonstrates that the yield of VLP antigen from the BEVS is greatly reduced for ORF2b when compared to ORF2a despite greater than 90% nucleotide and amino acid identity. An analysis of protein expression in SF+ cells revealed that ORF2b displayed a low solubility phenotype upon cell lysis which may contribute to the decreased ORF2 VLP yield observed with the BEVS. Mutational analysis of ORF2b found that replacement of the arginine at position 63 with threonine, as is at position 63 of ORF2a, dramatically increased yields of ORF2b VLPs from the BEVS. Further investigation found that the presence of charged or aromatic amino acids at

position 63 of ORF2b also resulted in decreased VLP yields. In addition, we demonstrated that the mutated protein, ORF2b R63T induced protective efficacy against virulent PCV2b challenge in young pigs. Similar to previously published studies, there was no significant difference in efficacy between the ORF2b vaccine and an ORF2a vaccine.

The BEVS is an ideal system for commercial production of PCV2 vaccines given the robust expression and assembly of PCV2a ORF2 and reliable scalability of the expression system [29]. Currently, the BEVS is utilized commercially to manufacture three PCV2a-based vaccines; Ingelvac CircoFLEX® (Boehringer Ingelheim), Circumvent® G2 and Porcilis® PCV (Merck). Recently, new USDA licensing regulations have been implemented to allow for shortened development timelines for new PCV2 vaccines based on the BEVS production platform [30]. It is likely that the scenario of low yields of VLP obtained from novel, yet closely related ORF2 proteins may occur in the future, which could potentially result in prolonged development timelines leading to delays in the availability of new vaccines in the event of the emergence of vaccine-resistant PCV2 strains. Therefore, the findings in this report are significant in that they highlight a potential problem for future BEVS-produced PCV2 vaccines and describe a solution to overcome this problem.

The low solubility phenotype observed for ORF2b after expression in insect cells could be the result of a variety of factors including protein misfolding and/or incorporation into larger molecular weight structures. Misfolding of proteins, especially those expressed from strong promoters such as the baculovirus polyhedrin promoter, can lead to formation of large aggregates of partially folded protein that are removed after centrifugation. As evolutionary pressure has selected for arginine at position 63 of ORF2 for PCV2b BDH and other strains of the PCV2b genotype, swine cells must contain a suitable molecular environment for proper folding of this

protein. The potential for ORF2 misfolding in insect cells may be the result of differences in the molecular environment between insect cells and swine cells, including the available repertoire of chaperone proteins.

Alternatively, ORF2b may fold properly but interact with itself or host macromolecular structures that pellet during centrifugation at 20,000 x g leading to an observed low solubility phenotype. A recent report demonstrates that release of a BDH-like PCV2b from infected porcine intestinal cells is significantly increased when the cells are treated with the F-actin inhibitor, cytochalasin D, suggesting that PCV2 may interact with the cytoskeletal network in a manner that is counter-productive to viral release [31]. As long F-actin filaments are known to pellet upon clarification of Triton cell extracts by centrifugation, a potential interaction between PCV2 BDH ORF2 and F-actin or F-actin binding proteins could result in a low solubility phenotype [32] . It's unclear whether the low solubility phenotype observed for ORF2b is confined to insect cells or if it occurs in swine cells. In addition, the observations made here are for recombinant ORF2 lacking the genomic DNA that may have a stabilizing effect on capsid formation. Further investigation into this area is needed to determine if the low solubility phenotype observed for ORF2b in insect cells plays a role in PCV2 replication in its natural host.

From the data it is clear that the low solubility phenotype observed for ORF2b does not completely account for the loss of VLP yield as the ORF2b R63T mutant retains the low solubility phenotype yet yields dramatically increased levels of VLP in the harvest supernatant. These data suggest that the R63T mutation may facilitate more efficient assembly of VLPs in the insect cell or that assembled ORF2b VLPs may be more stable when arginine is removed from this position. Further investigation in this area is needed to determine the mechanism of the increased VLP yield demonstrated by the ORF2b R63T mutant.

Taken together, the findings detailed in this report demonstrate that generation of ORF2 VLPs from emerging strains of PCV2 using the BEVS may be problematic, potentially leading to extended development timelines for new products. Our findings present a method for overcoming this problem which results in increased yields of immunogenic VLPs, thereby facilitating more rapid development of novel VLP-based PCV2 vaccines in the future.

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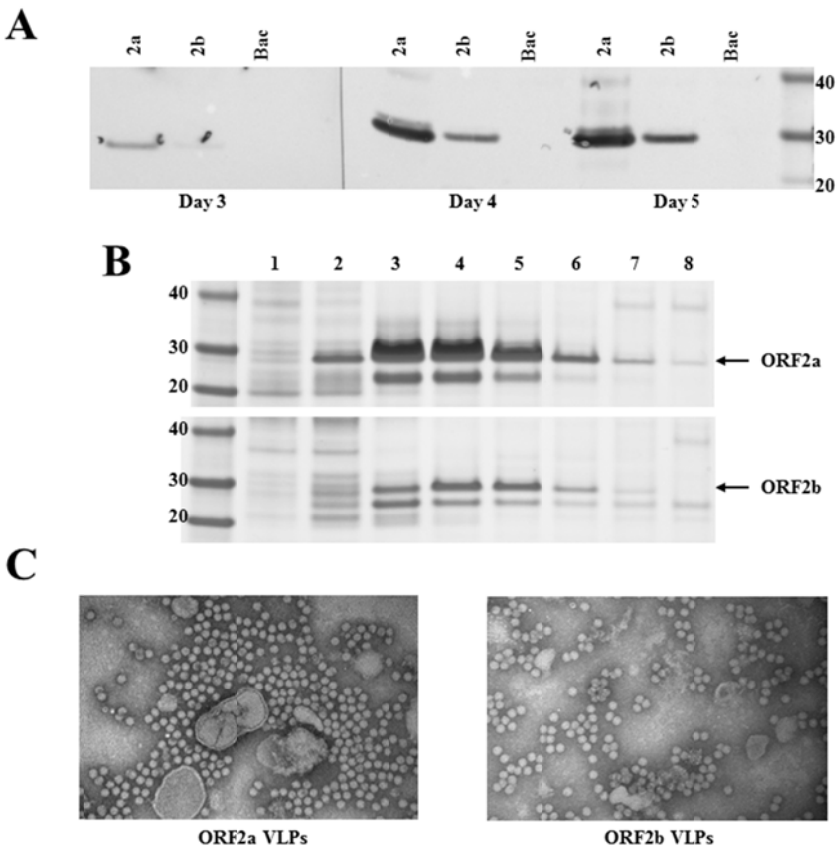


Fig 1. Expression of ORF2b and VLP formation. (A) Recombinant baculovirus encoding ORF2a (2a), ORF2b (2b) or mock insert (Bac) were used to infect SF+ cells at 0.1 MOI. Culture supernatants from recombinant baculovirus-infected SF+ cells were evaluated for PCV2 ORF2 by Western blot using PCV2-specific purified swine IgG at multiple timepoints during infection. Culture supernatants for day 5 were centrifuged at 100,000 x g for 2hrs and the resulting pellets reuspended and separated on a sucrose gradient. (B) Sucrose gradient fractions 1-8 of 12 containing ORF2a or ORF2b were separated by SDS-PAGE and stained for total protein. (C) The fractions containing ORF2a or ORF2b were pooled, processed, and analyzed for VLPs by TEM.

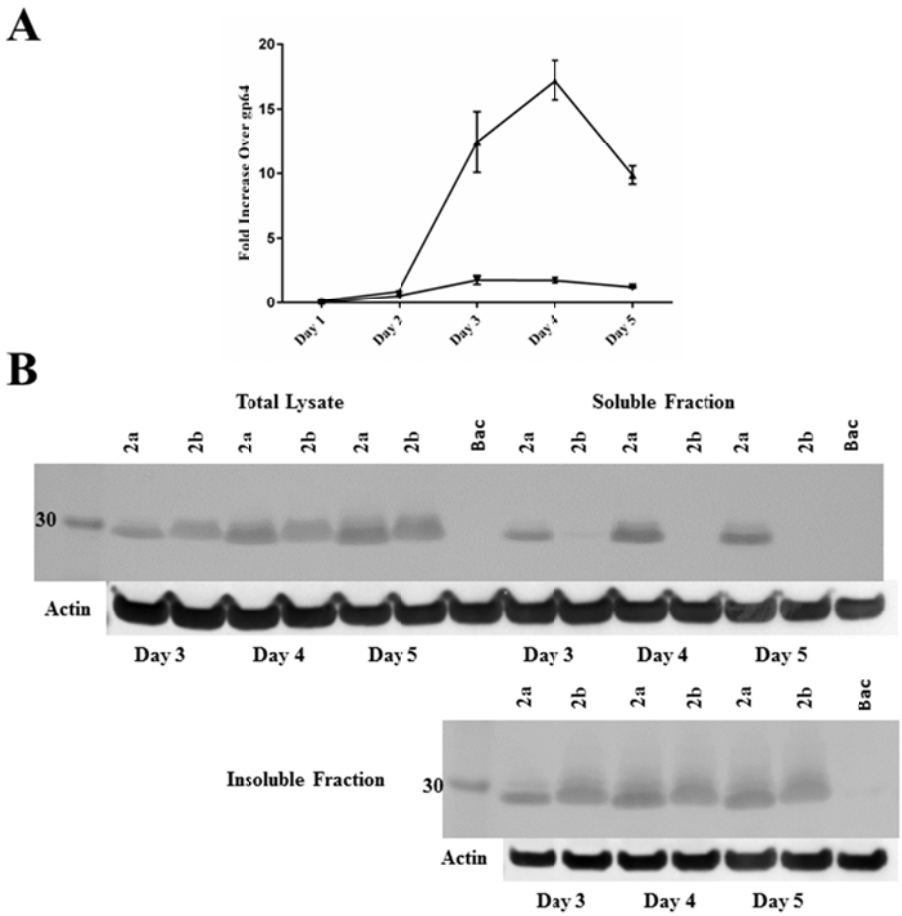


Fig 2. Comparison of ORF2a and ORF2b transcription and intracellular protein levels. Recombinant baculovirus encoding ORF2a (2a), ORF2b (2b) or mock insert (Bac) were used to infect SF+ cells at 0.1 MOI and cells were harvested daily. (A) Cell pellets were processed for total RNA and transcripts were detected using a qRT-PCR assay. Relative transcript levels for ORF2a (squares) and ORF2b (triangles) were calculated using the $2^{-\Delta\Delta CT}$ method and are presented as fold-increase over baculovirus gp64. (B) Alternatively, cell pellets were lysed with Triton X-100 and subsequently fractioned into soluble and insoluble fractions by centrifugation. Samples before (total lysate) and after fractionation (soluble fraction and insoluble fraction) were evaluated by Western blot with PCV2-specific purified swine IgG or anti-actin antibodies.

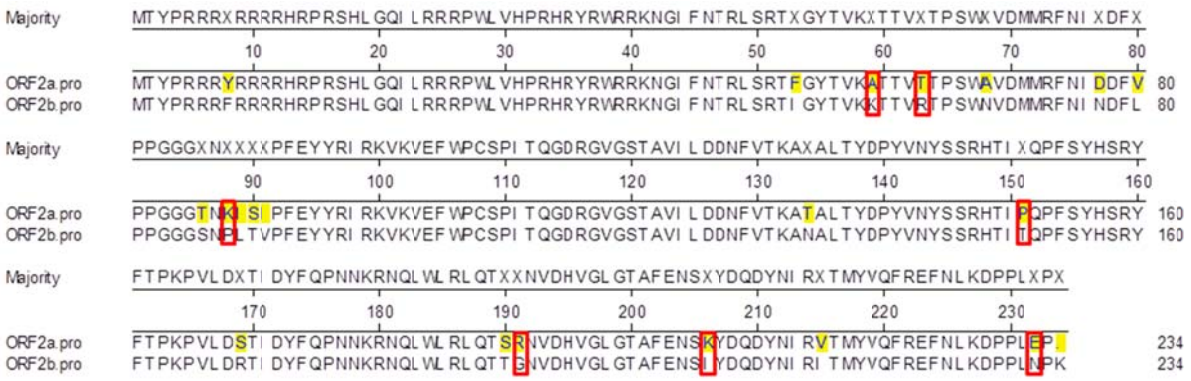
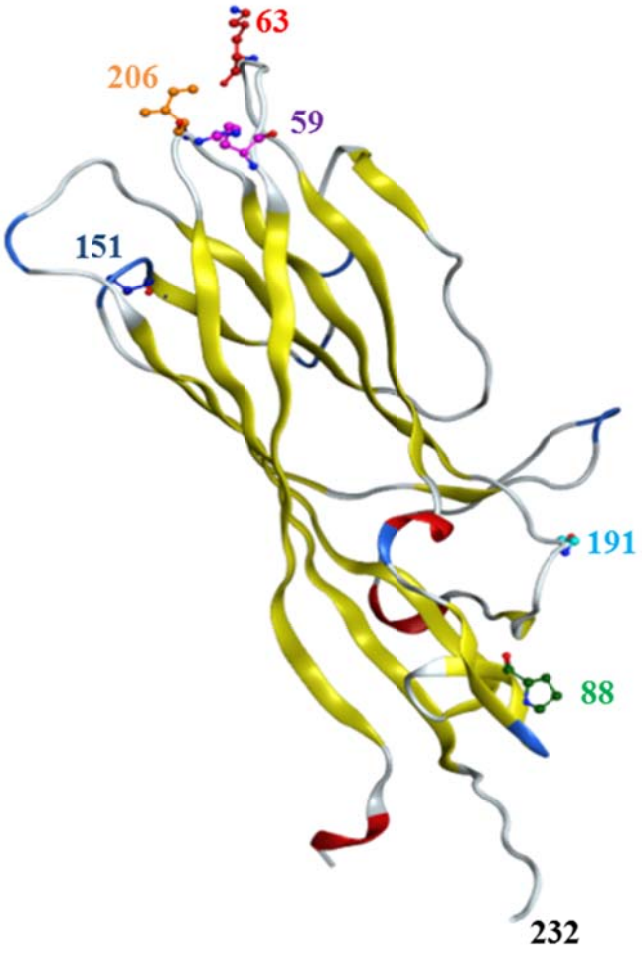


Fig 3. Alignment of ORF2a and ORF2b amino acid sequences. Sequences were aligned by the ClustalW method using MegAlign software version 11.1. Yellow highlighted amino acids represent differences in ORF2a when compared to ORF2b. Red boxes indicate the positions where ORF2a and ORF2b contain amino acids with dissimilar properties.



Position	ORF2a	ORF2b
59	A	K
63	T	R
88	K	P
151	P	T
191	R	G
206	K	I
232	E	N

Fig 4. PCV2 ORF2 monomer structure with postions of identified amino acid differences. Structure image generated from Protein Data Bank ID 3R0R using Molecular Operating Environment (MOE) 2013 software.

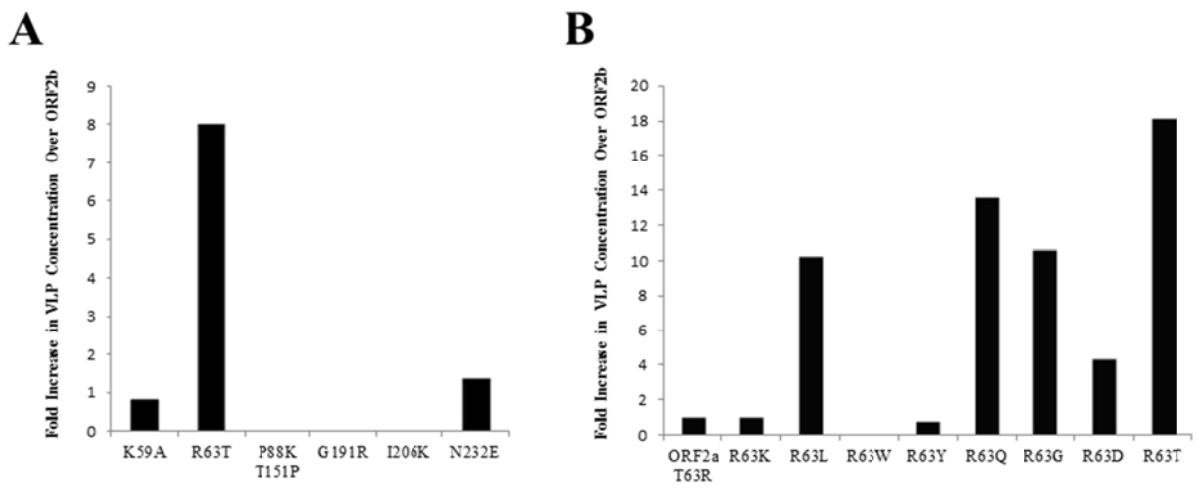


Fig. 5. VLP yield of mutant ORF2b baculovirus constructs. Culture supernatants from baculovirus-infected SF+ cells for each construct were processed by centrifugation at 100,000 x g. Resuspended pellets were separated on a sucrose gradient and ORF2-containing fractions were pooled and quantitated by gel densitometry. A sample of each pool was evaluated by TEM to confirm the presence of VLPs. The concentration of VLP in each pool as determined by gel densitometry was used to calculate the VLP concentration in the culture supernatant for each construct. Results are presented as fold increase in VLP concentration over ORF2b. (A) Mutant ORF2b constructs containing the corresponding ORF2a amino acid at positions 59, 63, 88, 191, 206, and 232. (B) Mutant ORF2a construct containing arginine at position 63 and mutant ORF2b constructs with similar and non-similar amino acid substitutions at position 63.

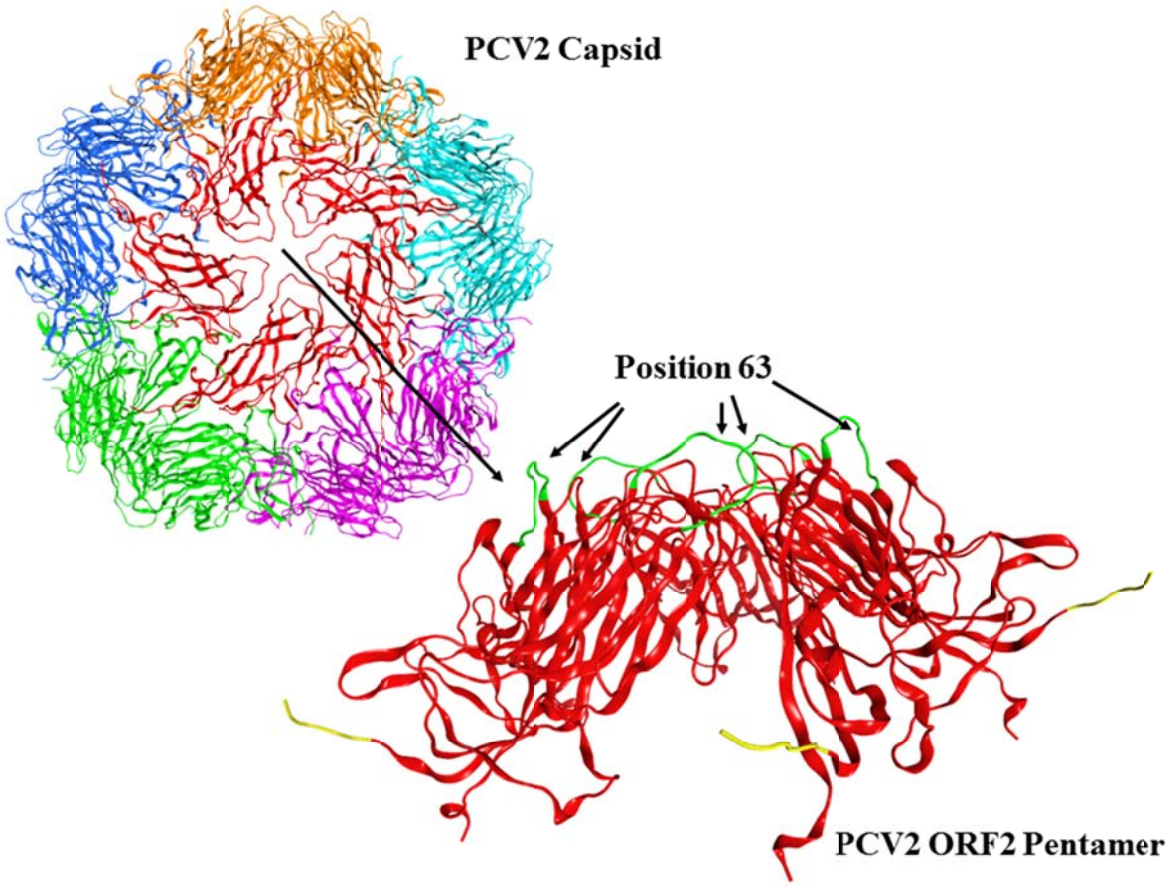


Fig 6. Orientation of position 63 of PCV2 ORF2. Colored sections of capsid image indicate ORF2 pentamer units. The green loops on the image of the red pentamer unit represent the loop between β-strands B and C as designated by Khayat et. al. 2011 while the yellow regions indicate the C-terminal region. Structural images generated from Protein Data Bank ID 3R0R biological assembly using Molecular Operating Environment (MOE) 2013 software.

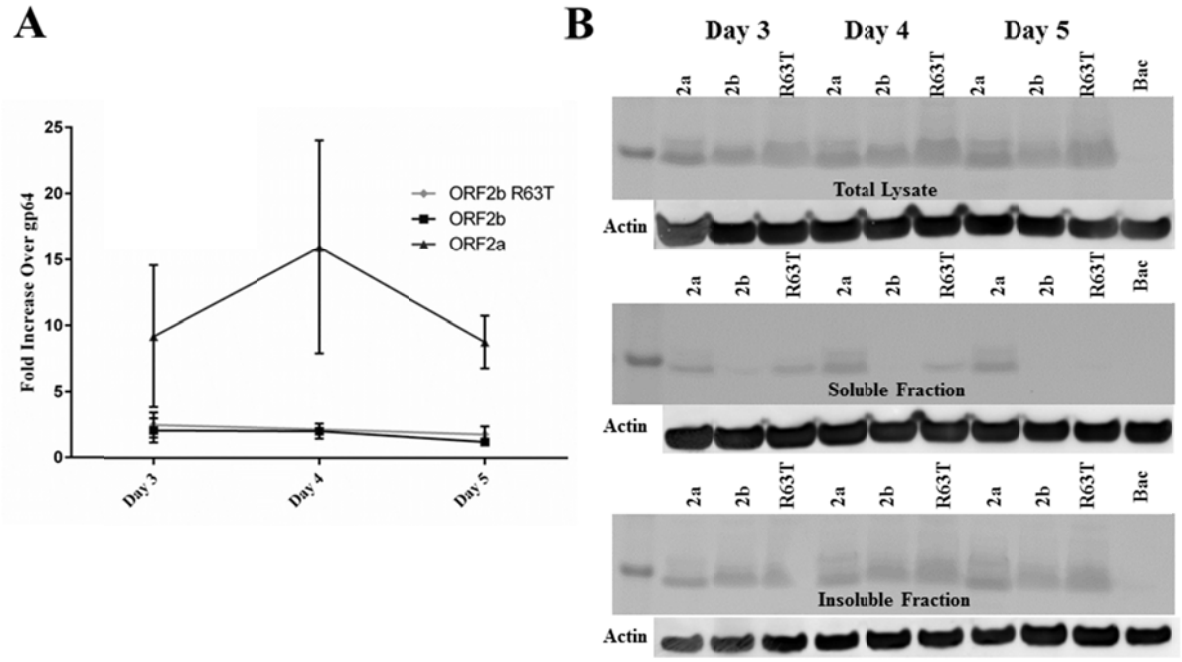


Fig 7. Comparison of ORF2a, ORF2b, and ORF2b R63T transcription and intracellular protein levels. Recombinant baculovirus encoding ORF2a (2a), ORF2b (2b), ORF2b R63T (R63T) or mock insert (Bac) were used to infect SF+ cells at 0.1 MOI and cells were harvested daily. (A) Cell pellets were processed for total RNA and transcripts were detected using a qRT-PCR assay. Relative transcript levels for ORF2a, ORF2b, or ORF2b R63T were calculated using the $2^{-\Delta\Delta CT}$ method and are presented as fold-increase over baculovirus gp64. (B) Alternatively, cell pellets were lysed with Triton X-100 and subsequently fractioned into soluble and insoluble fractions by centrifugation. Samples before (total lysate) and after fractionation (soluble fraction and insoluble fraction) were evaluated by Western blot with PCV2-specific purified swine IgG or anti-actin antibodies.

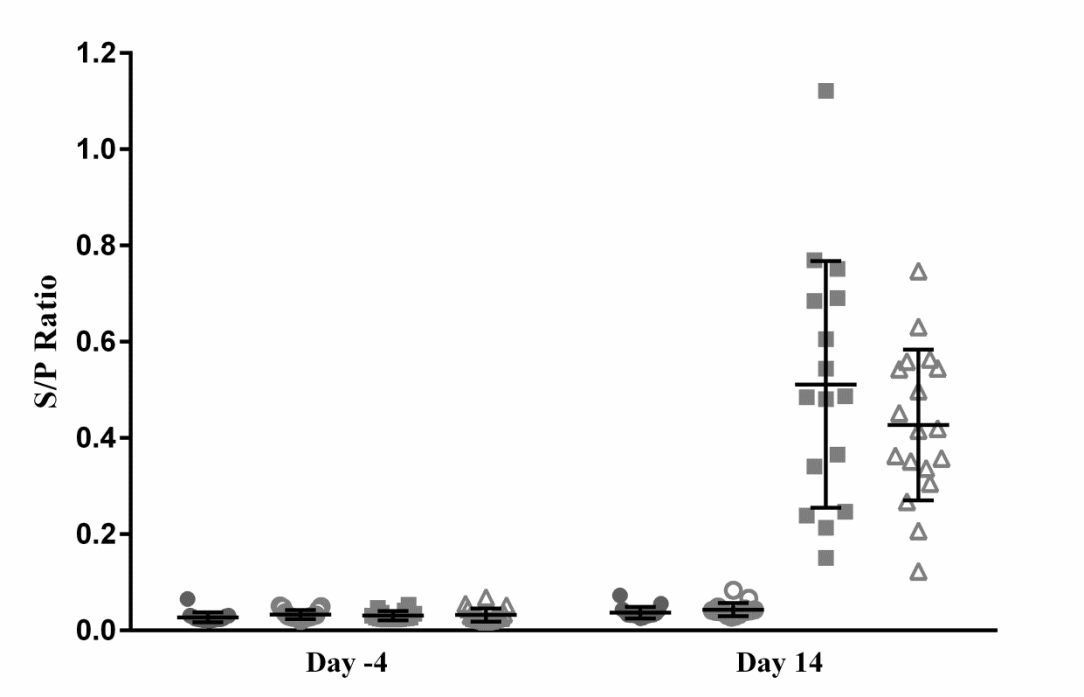


Fig 8. Humoral response to PCV2 vaccines measured by ELISA. Serum samples were collected prior to and 14 days after vaccination. Pigs were vaccinated with ORF2a or ORF2b R63T vaccines or left untreated. The presence of anti-immunogen IgG was detected by ELISA on plates coated with purified ORF2a or ORF2b R63T VLPs. S/P ratios +/- SD are presented for untreated control (circles), ORF2b R63T-vaccinated (squares), and ORF2a-vaccinated (triangles) pigs with bars depicting group mean and standard deviation. S/P ratios for open symbols were determined on ORF2a VLP-coated plates and those for closed symbols were determined on ORF2b R63T VLP-coated plates.

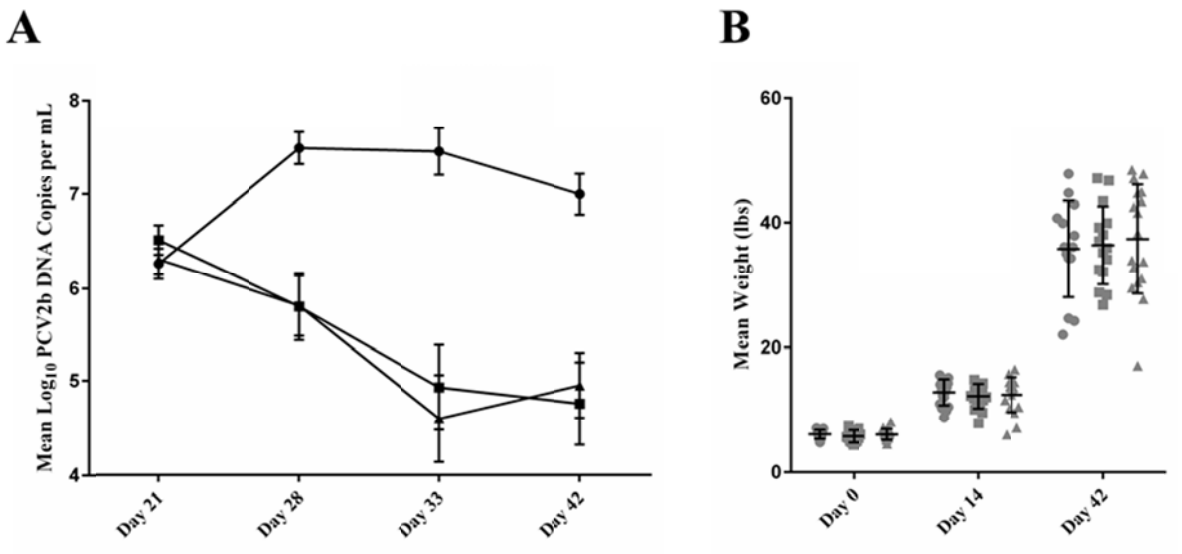


Fig 9. Detection of PCV2b DNA in the serum and weight gain after challenge. Pigs were vaccinated with ORF2a or ORF2b R63T vaccines or left untreated at Day 0 and subsequently challenged at day 14. (A) PCV2b DNA was detected in serum samples with a real-time PCR assay utilizing PCV2 specific primers and a PCV2b-specific probe. Copy numbers per milliliter of serum were $\log_{10}$ transformed and the group means +/- SEM for the untreated control (circles), ORF2b R63T-vaccinated (squares), and ORF2a-vaccinated (triangles) groups are presented for each time point. (B) Pig weights at vaccination (day 0), challenge (day 14), and necropsy (Day 42) with group mean +/- SD depicted by the bars. Symbols represent the same groups as in (A).

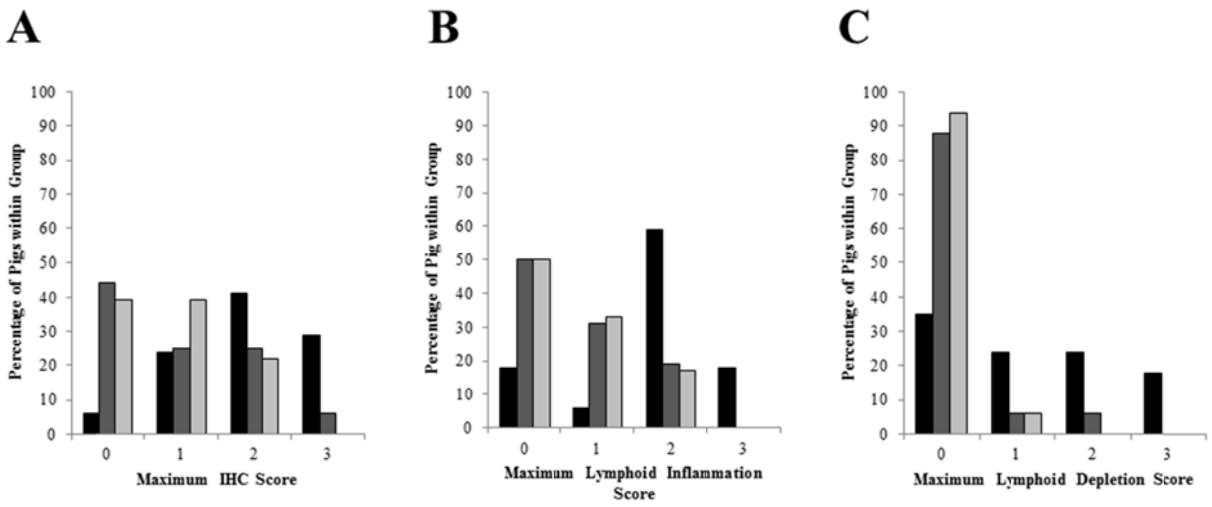


Fig 10. Maximum lymphoid lesion scores at necropsy. Pigs were vaccinated with ORF2a or ORF2b R63T vaccines or left untreated and subsequently challenged at day 14. Four weeks after challenge, pigs were euthanized and lymphoid tissue samples were evaluated for granulomatous inflammation, lymphoid depletion, and the presence of PCV2 antigen by IHC. A maximum severity score was assigned to each pig for (A) IHC, (B) lymphoid inflammation, and (C) lymphoid depletion based on the highest score for each category in any of the four tissues evaluated (tonsil, ILN, TBLN, and MLN). The data presented here are the percentage of pigs in the untreated control group (black bars), ORF2b R63T-vaccinated group (dark grey bars), and the ORF2a-vaccinated group (light grey bars) with the indicated maximum severity score.

Table 1. Vaccination/challenge study design.

Group	# of Pigs	Day 0 Vaccination	Days 11 & 17	Day 14	Day 42
1	18	N/A	KLH/ICFA Treatment	PCV2b Challenge	Necropsy
2	18	ORF2b R63T	KLH/ICFA Treatment	PCV2b Challenge	Necropsy
3	18	ORF2a	KLH/ICFA Treatment	PCV2b Challenge	Necropsy

Table 2. Primer and probe sets for qPCR and qRT-PCR.

Target Gene	Oligo	Sequence 5' - 3'	Conc. (µM)	Reference
S. frugiperda 28s	Primer 1	GCTGGCTTGATCCAGATGTTCAG	0.5	Salem et. al. 2014 (Primer 1)
	Primer 2	GGTAACTTTTCTGGCACCTCTTGC	0.5	
	Probe	TEX615-CGATAGGCCGTGCTTTCGCAGTCC-BHQ_2	0.1	
Baculovirus gp64	Primer 1	ACGGTCACGTTGATGGGGTT	0.5	N/A
	Primer 2	TGAAGCGGCAGAATAACAATCACT	0.5	
	Probe	Cy5-CTCGTCCGTGTCGTCCTGGCACTC-BHQ_2	0.1	
PCV2a ORF2	Primer 1	GTAACGGGAGTGGTAGGAGAA	0.125	Brunborg et. al. 2004
	Primer 2	GCCACAGCCCTAACCTATGAC	0.125	
	Probe	FAM-ATGTAAACTACTCCTCCCGCCATACAATC-BHQ_1	0.5	
PCV2b ORF2	Primer 1	GCAGGGCCAGAATTCAACC	0.25	Opriessnig et. al. 2010
	Primer 2	GGCGGTGGACATGATGAGA	0.25	
	Probe	FAM-CTCAAACCCCCTCACTGTGCCC-BHQ_1	0.1	

Table 3. Observed mitigated fractions with bootstrap 95% confidence limits.

Contrast	2.5 Percentile (Lower 95% CL)	Observed Mitigated Fraction	97.5% Percentile (Upper 95% CL)
Max. of Lymph Node Immunohistochemistry: ORF2a vs Untreated	0.4375 (43.75%)	0.686 (68.6%)	0.9375 (93.75)
Max. of Lymph Node Immunohistochemistry: ORF2b vs Untreated	0.4 (40%)	0.617 (61.7%)	0.8298 (82.98%)
Max. of Lymph Node Depletion: ORF2a vs Untreated	0.4259 (42.59%)	0.627 (62.7%)	0.8571 (85.71%)
Max. of Lymph Node Depletion: ORF2b vs Untreated	0.4043 (40.43%)	0.511 (51.1%)	0.6383 (63.83%)
Max. of Lymph Node Inflammation: ORF2a vs Untreated	0.4815 (48.15%)	0.608 (60.8%)	0.7778 (77.78%)
Max. of Lymph Node Inflammation: ORF2b vs Untreated	0.4545 (45.45%)	0.617 (61.7%)	0.7872 (78.72%)

CHAPTER 4: GENERAL CONCLUSIONS

The work presented in this dissertation demonstrates the versatility of the baculovirus expression vector system (BEVS) in the development of vaccines against viruses associated with the porcine respiratory disease complex (PRDC). Swine influenza virus (SIV) and porcine circovirus type 2 (PCV2) are two important swine pathogens and in the case of SIV, a potential human pathogen. The protective antigens for these two viruses require distinct post-translational processing pathways as hemagglutinin of SIV is a type 1 glycoprotein and ORF2 of PCV2 is a capsid protein that assembles into virus-like particles (VLPs). Although vaccines are currently available to combat these pathogens, the antigenic variability displayed by SIV and PCV2 necessitates continued development of efficacious vaccines.

Detailing the evaluation of three experimental vaccines against swine influenza virus (SIV) using recombinant hemagglutinin antigens, the first study showed that the BEVS platform may be used to facilitate rapid development and licensing of novel SIV vaccines to address the increasingly diverse landscape of circulating viral strains in pigs. Vaccines containing H1 hemagglutinin prepared as an IgG fusion protein, displayed in the baculovirus envelope, or displayed in an enveloped FeLV gag VLP using the BEVS were evaluated for efficacy in the face of a H1N1 SIV challenge. Each vaccine was found to be immunogenic and provide protective efficacy against the H1N1 SIV challenge. As vaccination of pigs for SIV typically occurs at a young age, the performance of these vaccines in the face of maternal antibody is an important question that remains unanswered. Additionally, the duration of immunity elicited by these vaccines must be determined and compared to the inactivated whole virus vaccines currently available.

The results of the second study uncovered an important problem for the future development of VLP-based PCV2 vaccines using the BEVS. Yields of VLP antigen from baculovirus encoding an ORF2 protein from emerging strain PCV2b BDH were found to be considerably reduced when compared to VLP antigen generated from baculovirus encoding a PCV2a ORF2 (ORF2a) protein which is currently the basis for two highly effective PCV2 vaccines. Further investigation demonstrated that an arginine residue at amino acid position 63 was responsible for the low VLP yield and could be replaced with a number of non-basic amino acids to increase PCV2b BDH ORF2 (ORF2b) VLP yields to commercially viable levels. Additionally, the modified ORF2b VLP antigen was found to retain its immunogenic and efficacious properties in a vaccination/challenge study. As the BEVS is an important commercial production platform for PCV2a ORF2 VLP-based vaccines for PCV2, the findings detailed in the second study are significant in that they describe a solution for potential problems that may be encountered for future development of VLP-based vaccines using the BEVS. Although the available vaccines based on PCV2a ORF2 antigen are considered effective against currently circulating strains of PCV2, subclinical infections are common and novel strains continue to surface in vaccinated swine herds. Given this situation, it would be interesting to test whether a combination vaccine containing VLP antigens from multiple PCV2 strains may be more effective in eliminating subclinical infections and thereby reducing the incidence of novel isolates.

In conclusion, the research contained in this dissertation describes successes and challenges in the development of veterinary vaccines utilizing the BEVS, paving the way for BEVS-based SIV vaccines and addressing a potential problem for future BEVS-based PCV2 vaccines for swine.

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